Universidade de Aveiro Departamento de Biologia

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Biomonitorização de aves em recuperação: um estudo de longo termo

Biomonitorization of birds under recovery: a long term study

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Dissertação apresentada à Universidade de Aveiro para obtenção do grau de Mestre em Biologia Aplicada – Ramo de Ecologia, Biodiversidade e Gestão dos Ecossistemas, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro, Investigadora auxiliar do Departamento de Biologia e CESAM (Centro de Estudos do Ambiente e do Mar) da universidade de Aveiro, e co-orientação da Doutora Marta Sofia Soares Craveiro Alves Monteiro dos Santos, Investigadora de Pós-Doutoramento do Departamento de Biologia e CESAM da Universidade de Aveiro.

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palavras-chave aves, ecotoxicologia, biomarcadores, genotoxicidade, neurotoxicidade.

resumo

O uso de aves, em particular aves aquáticas, como bioindicadores de qualidade ambiental tem vindo a ser aplicado em estudos de diversos tipos de ecossistemas. São vários os atributos que tornam as aves espécies de interesse na biomonitorização ambiental, como a sua abundância, facilidade de encontrar no campo e particularmente sensibilidade a contaminantes ambientais, nomeadamente toxinas e contaminantes bioacumuláveis. Nos últimos anos, uma parte significativa dos estudos de biomonitorização realizados em Portugal tem-se focado essencialmente em organismos de níveis tróficos inferiores (ex. larvas, crustáceos e bivalves), mas pouca atenção tem sido dada a organismos de níveis tróficos superiores, tais como mamíferos ou aves.

O presente trabalho teve como principais objetivos: (i) avaliar a exposição ambiental das aves portuguesas a contaminantes ambientais, em particular de aves aceites para reabilitação em centros de recuperação de animais selvagens, (ii) esclarecer se esses fatores podem ou não conduzir à doença das aves e influenciar a sua recuperação, e (iii) entender se o uso de ferramentas ecotoxicológicas pode ou não ser uma mais-valia na monitorização e recuperação dessas mesmas aves. No sentido de esclarecer estas questões foram avaliados marcadores de neurotoxicidade e genotoxicidade em aves aquáticas das ordens Ciconiiformes, Charadriiformes e Pelecaniformes. Na primeira parte deste trabalho (análise de biomarcadores de neurotoxicidade), e em dois estudos independentes, foi feita a caracterização das colinesterases presentes no plasma da cegonha branca (*Ciconia ciconia*), garça-real (*Ardea cinerea*) e do ganso-patola (*Morus bassanus*) e avaliada a reativação da colinesterase (ChE) presente no cérebro da gaivota-argêntea (*Larus michahellis*). A impossibilidade da interligação entre estes estudos e uma exploração mais detalhada deve-se à (in)disponibilidade de amostras/aves. Na segunda parte deste trabalho, para a avaliação de efeitos genotóxicos, foi analisada a frequência de micronúcleos e outras anomalias nucleares em eritrócitos de cegonha branca (*Ciconia ciconia*), garça-real (*Ardea cinerea*), garça-vermelha (*Ardea purpurea*) e garça-branca-pequena (*Egretta garzetta*).

A pseudocolinesterase (PChE) foi o principal tipo de colinesterase identificada no plasma de *C. ciconia*, *A. cinerea* e *M. bassanus*. Por sua vez, nos ensaios de reativação observou-se um aumento significativo (superior a 50%) na atividade da colinesterase presente no cérebro de *L. michahellis*, sugerindo exposição prévia destes indivíduos a anticholinesterásicos .

Estes resultados sugerem que as aves portuguesas poderão estar expostas a diferentes graus de contaminação ambiental, podendo esta contaminação deteriorar a saúde das aves. O uso de ferramentas ecotoxicológicas na monitorização de aves em reabilitação afigura-se-nos, por isso, como sendo uma mais-valia pois permitirá identificar de forma mais precoce sinais fisiológicos de toxicidade e assim executar uma avaliação mais criteriosa do estado físico das aves. Para além disso, a monitorização através da utilização deste tipo de biomarcadores poderá permitir seguir a potencial recuperação dessas aves.

keywords birds, ecotoxicology, biomarkers, genotoxicity, neurotoxicity.

abstract

Birds, including waterbirds, have been used as bioindicators of environmental quality in a broad range of ecosystems. Amongst other attributes, their abundance, conspicuousness and sensibility to environmental contaminants, including bioaccumulative chemicals and toxins, are some of the characteristics that make them key species in environmental biomonitorization. Over the past years a significant part of the Portuguese biomonitoring studies has focused on organisms at lower trophic levels (e.g. larvae, crustacean and mollusks), but failed to address contaminants' effects upon organisms at higher trophic levels such as mammals or birds.

The present study aims were to: (i) assess the exposure of Portuguese birds to environmental contaminants, in particular birds accepted for rehabilitation in wildlife recovery centres, (ii) clarify if these factors could lead to birds illness and influence their recovery, and (iii) understand if ecotoxicological tools can help and be useful tools in the future to monitor and aid bird's recovery. In order to address these issues, it was assessed markers of neurotoxic and genotoxic exposure in aquatic birds from the orders Ciconiiformes, Charadriiformes and Pelecaniformes. In the first part of this study (the analysis of neurotoxic markers), it was characterized the cholinesterase form present in plasma of the white stork (*Ciconia ciconia*), grey heron (*Ardea cinerea*) and northern gannet (*Morus bassanus*) and the cholinesterase (ChE) reactivation in brain of the yellow-legged gull (*Larus michahellis*) was assessed. The impossibility of interconnection between these studies and a more detailed exploration was due to the (un)availability of samples/birds. In the second part of this work, for the assessment of genotoxic effects, the frequency of micronucleus and other nuclear abnormalities was analysed in erythrocytes of the white stork (*Ciconia ciconia*), grey heron (*Ardea cinerea*), purple heron (*Ardea purpurea*) and the little egret (*Egretta garzetta*).

Pseudocholinesterase (PChE) was the main cholinesterase present in plasma of *C. ciconia*, *A. cinerea* and *M. bassanus*. Moreover, cholinesterase activity in brain of *L. michahellis* was found to get reactivated at a significant extent (activity increase in 50%), suggesting a previous exposure of these individuals to anticholinesterase agents. High levels of genotoxic damage were also observed in the species of Ciconiiformes studied, with these values varying significantly between different years and geographical origins ($P < 0.05$).

These results suggest that Portuguese birds might be exposed to different levels of environmental contamination and that this contamination may impair birds' health. The use of ecotoxicological tools seems to be a very promising way to help monitor and aid bird's recovery as it will probably allow screening for early physiological signs of toxicity, therefore enabling a more insightful evaluation of birds' health condition. Moreover, the use of these types of biomarkers may allow to monitor the potential rehabilitation of these birds.

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Chapter 1

General Introduction, Objectives and Relevance of the Work

1. 1. General introduction

Since the time of Neanderthal Man, when fire was discovered resulting into the release to the environment of soot and polycyclic aromatic hydrocarbons from combustion processes, pollution has always been present in human civilizations [1]. Environmental pollution as we know today, though, only arisen after industrial revolution. The emergence of industries and consequent consumption of coal and other fossil fuels, allied to the tremendous demographic boost, leaded to an unprecedented increase on volume of both industrial and human wastes, disposed off through flagrant release into the environment [2]. It wasn't however until the 1950s to 1970s that first concerns over environmental pollution began to draw public attention.

Prompted up by Rachel's Carson "The Silent Spring", published in 1962, the crescent evidences of the harmful effects of chemicals upon wildlife spurred the approval of legislation aimed at regulating and limiting the release of chemical wastes into the environment [2]. Impelled also by these same concerns, it was in the late 1960s that the modern environmental toxicology and its subfield of ecotoxicology arisen.

Contemporarily understood as being the science that relates the harmful effects of chemical agents in living beings, the term "ecotoxicology" was firstly introduced in 1969 by René Truhaut as the branch of toxicology that studied the toxic effects caused by natural or synthetic pollutants on the living systems [3]. A multidisciplinary science, ecotoxicology recognizes that the toxicity induced by contaminants is originated at the molecular level, by altering essential cellular mechanisms, which in turn results in a hierarchical cascade of changes from physiological processes to ecosystem function [4]. Being its main aim to understand and anticipate the distribution path of pollutants in the environment (as well as the ecological effect at them associated), ecotoxicology employs ecological parameters to access toxicity, providing a wide range of valuable tools in ecosystems monitoring and therefore playing a crucial role in the management, restoration and conservation of natural systems [3].

1.1.1. Wildlife toxicology: a field within ecotoxicology

Wildlife toxicology is the branch of ecotoxicology focused on the study of the effects of environmental contaminants (e.g. survival, health, reproduction) upon amphibians, reptiles, mammals and birds [5,6]. Unlikely other scientific fields whose knowledge is acquired through deliberate hypothesis testing and discovery, wildlife toxicology has been

driven by chemical use and misuse, catastrophic animal poisonings, ecological mishaps and the research conducted in the field of human toxicology [6].

Recognition of deleterious effects of contaminants upon wildlife can be traced back to the end of industrial revolution, when initial reports of lead poisoning from ingestion and retention of spent shot on pheasant *Phasianus colchicus* (England, 1876) and waterfowls (USA, 1894) appeared [6]. Lead toxicity was manifested by emaciated birds with paralyzed legs and feet, with feet being held similarly to the "drop-hand" condition observed in lead-poisoned humans. [7]. Increased awareness of environmental problems and endangered species protection was gained during the early twentieth century. With expanded production and use of petroleum right after World War I, thus leading to an increase of the oil transported across the ocean, first oil spills occurred resulting in the death of numerous marine birds [6,8]. As reports of harmful effects of contaminants' upon birds and other wildlife populations continued to drawn public and scientific attention, prompting the development of a wide range of tools to assess exposure and effects of contaminants on impacted populations, wildlife toxicology made a standing.

The use of improved analytical, molecular and biochemical tools allied to the scientific advances made in ecology and other complementary fields has also greatly contributed to the development of wildlife toxicology. By 1950s, first bioaccumulation studies were developed, aiming to assess organochlorine pesticides burdens in wildlife [6]. On this same decade, first studies that aimed to relate lethality and pathology data of contaminants to tissue concentrations on wildlife were published. Earliest work, by Coburn et al. [9], tested phosphorus tissue concentrations indicative of exposure in both black and mallard duck (*Anas rubripes* and *Anas platyrhynchos,* respectively), through a combination of field investigations and dosing trials. Investigation of toxicity thresholds continued, and towards the latter half of 1970s first controlled exposure studies were initiated in game and captive wild bird species to determine sublethal responses (e.g. reproduction, behaviour, biochemical indicators of exposure, physiological and endocrine function) of pesticides, metals and crude oil [6].

As methodologies used in wildlife toxicology were successively improved, in field studies, it was demonstrated for the first time that pesticide use and agricultural practices could also affect indirectly farmland birds' population by altering habitat vegetation and availability of preferred insects, thus reducing dramatically their food supplies [10]. Back in 1990s, diagnose of contaminant exposure and effects was further improved by the development of new molecular assays (e.g. markers of genetic damage and oxidative stress) and use of nondestructive sampling methodologies [6,11]. In more recent years,

other tools to assess contaminant exposure and effects upon wildlife have been developed recurring to techniques such as DNA fingerprinting, Polymerase chain reaction (PCR) and microarrays, allowing to acquire information more detailed on the effects of contaminants beyond individual and cellular levels [5]

It is a fact that the development of new and improved sophisticated tools to assess exposure, effects of contaminants upon wildlife populations has greatly contributed to the development of wildlife toxicology as a science, as well as in the protection of endangered wildlife species. In spite of this, however, the ability to predict and prevent ecological mishaps remains nowadays still very limited, which can be partly attributable to the logistical difficulties of studying wild animal populations and/or extrapolating data generated in laboratory [6]. The varying sensitivity of animals at the species and even individual level poses a problem, being difficult to assess contaminants' concentration-effects on wildlife; moreover, the fact that multiple contaminants may coexist within a natural system enhances the probability of occurring combined effects, which may act as a confounding factor in the prediction and/estimation of contaminants' effects on wildlife and their supporting habitat.

1.1.2. Perturbations in birds' populations: contaminants as a cause

Similarly to other vertebrates, avian exposure to environmental contaminants is dependent on contaminants' and habitats' physiochemical properties, life history characteristics (e.g. age, gender, size), and behavioural traits that are intrinsic to each bird species, among others [12]. Exposure routes in birds may occur through four pathways: ingestion, inhalation, dermal contact and maternal transfer.

Ingestion is the principal pathway of contaminant exposure in birds and may occur through direct intake of free chemicals (e.g. accidental ingestion of liquid formulations following spills, or through intentional poisoning by humans) or more commonly, indirectly, through ingestion of contaminated food (e.g. ingestion of prey items that have previously accumulated contaminants in their tissues), or water [13]. Dermal exposure and maternal transfer are also important routes of exposure in avian species and may occur through contact of the contaminant with the eyes and the skin of the legs and feet, or as a result of the transfer of pollutants from gravid females into eggs, respectively [12,14,15]. In regard of inhalation exposure, and even thought this route has been sparsely documented in the

literature, exposure occurs mainly through contact with compounds applied or released aerially or of volatile nature [12].

Due to their ability to adapt and thrive in disturbed habitats and, on the other hand, their sensitivity to numerous environmental pollutants, contaminant exposure on birds has been far more documented than in any other group of terrestrial vertebrates [12]. The first evidences of contaminant-effects relations in bird populations had its origin in the 1950s and 1960s, when the effects of organochlorine insecticides such as DDT were first noticed [16].

DDT was first synthesized as a controlling agent of malaria and typhus among civilians and troops during the second half of World War II, but its use was generalized to agricultural and domestic use back in 1945, leading to a widespread decline of birds of prey [17,18]. Within one year of use, nesting failures (e.g. failure of adults to return to traditional nest, to lay eggs, or failure of eggs to hatch) on Florida's subpopulation of bald eagle (*Haliaeetus leucocephalus*) were noticed by C. L. Broley [19]. A similar trend was reported by D. A. Ratcliffe in 1967, with avian population declines of peregrine falcon (*Falco peregrinus*), golden eagle owl (*Aquila chrysaetos*) and the european sparrow hawk (*Accipiter nisus*) being related to reproduction failures caused by an increase in eggshell thinning (which subsequently led to egg breakage and embryo death), starting immediately after DDT introduction in Britain [17,20]. It was only in 1970 that Ratcliffe was able to report a close relationship between the changes in eggshell thickness and the residual levels of DDT present in the eggs of 14 avian species [17]. These findings were in accordance with a previous study of Hickey and Anderson, in 1968, who also found a decrease of shell thickness in herring gull (*Larus argentatus*) associated with the increase of chlorinated hydrocarbon residues (such as DDE, a DDT metabolite) in eggs [17,21]. Further evidence of these initial reports were later substantiated and extended by similar works with other species and in other countries [17].

Another example of contaminants with clear effects upon bird populations is crude petroleum and its refined products. Adult birds can be affected by crude oil through a wide variety of routes, from ingestion to induced habitat degradation, but most frequently crude oil deleterious effects occur through external oiling. External oiling can lead to increased feather permeability, which may lead to hypothermia, drowning, ingestion through feather preening and habitat degradation [8]. Other effects reported in the literature in adult individuals include disease (e.g. gastrointestinal irritation, pneumonia), starvation, impairment of the function of the immune and nervous system and predation, which may occur as a consequence of direct ingestion of crude oil [8,22]. Birds' embryos have also

been found to be highly sensitive to crude oil and its derivates. For example, quantities of about 1-2 μL of some derivates of oil are sufficient to cause embryo death [8]; other effects listed in the literature include reduced hatchability and modifications of the yolk structure of eggs, as well as reduced incubation attentiveness and chick survival [23,24].

1.1.3. Waterbirds as indicators of environmental quality

Over the twentieth century, the concern over environmental changes has sparked the development of ecological indicators (e.g. concentration of pollutants in water, altered nutrient dynamics, taxa richness) to assess environmental health [25,26]. Within this context, bioindicators are measurable responses on living organisms (e.g. biological processes, species and communities composition) whose condition can be monitored to evaluate environment or ecosystem's integrity.

Birds, including waterbirds, have been used as bioindicators of environmental quality in a broad range of ecosystems [27-29]. Amongst other attributes, birds are abundant, conspicuous and functionally important components of ecosystems [30]. In addition, they are highly mobile and sensitive to both direct and indirect environmental changes (e.g. contamination, perturbation, duration of seasonal frequency); as top predators in both aquatic and terrestrial food webs, they are good indicators for bioaccumulative chemicals and toxins, as well as for the detection of diseases, being also key species for education and public awareness [27,30]. Finally, they're relatively easy and inexpensive to quantify and their basic ecology and habitats preferences are commonly well established [30].

Traditionally, risk assessment studies with natural bird populations have been focused either in the study of the relation between organisms' lethality and pathology data from contaminants to tissue concentrations on wildlife, or in the assessment of biomarker responses in individuals. In a broad sense, biomarkers can be understood as detectable responses of varying nature (e.g. molecular, cellular or physiological) that can be used to detect exposure, susceptibility and effect of chemical contaminants on living organisms. They can be classified as [31]:

- *Biomarker of exposure:* measures the interaction between a contaminant substance and a target molecule or cell that is measured in a compartment within an organism. Some of this type of biomarkers allows to measure the internal dose of the toxic compound or derived metabolites found for example in body fluids, organs or biological systems [32].

Numerous studies have addressed this kind of marker; some examples include the measurement of metals (e.g. mercury, arsenic, lead, selenium, etc.) or organic contaminants (e.g. polychlorinated byphenyls, organochlorine pesticides, etc.) burdens in feathers, internal tissues (e.g. liver), eggs and/or blood in the herring gull (*Larus argentatus*) [15], black-winged stilt (*Himantopus himantopus*) [33] and mourning doves (*Zenaida macroura*) [34].

- *Biomarker of effect:* evaluates biochemical, physiological, behavioural, or other alterations observed within an organism that, depending upon their magnitude, can be associated to a health impairment or disease. This kind of markers screens for changes (e.g. modifications on blood composition, changes in specific enzymatic activities) within an organism that can be translated as indicative of xenobiotic exposure [32].

An example of this type of biomarker is cholinesterase (ChE) activity. Cholinesterases are a family of esterase enzymes constituted by two forms: acetylcholinesterase (AChE; EC 3.1.1.7), which is mainly found at neuromuscular junctions and central nervous system, and pseudocholinesterase (PChE; EC 3.1.1.8) also denominated by butyrylcholinesterase (BChE), mainly found on liver and serum [35]. Inhibition of ChEs activity has been used as a primary indicator of exposure to organophosphate (OP) and carbamate pesticides (CB) in several bird species such as mallard duck (*Anas platyrhynchos*) [36], great blue heron (*Ardea herodias*) [37], glossy ibis (*Plegadis falcinellus*) [14] and great egret (*Nycticorax nycticorax*) [14,38]. Although organophosphorous and carbamate compounds can be less hazardous to the environment than organochlorine pesticides, as they have a short half-life thus do not tend to persist in animal tissues, some of these chemicals are extremely toxic for short periods of time right after their application [39]. OP and CB compounds exert their toxicity through inhibition of AChE, which leads to an accumulation of the neurotransmitter acetylthiocholine at synapses and neuroeffector junctions, leading to a cascade of effects from disruption of processes in the central nervous systems to death by paralysis [39,40]. Inhibition of PChE/BChE has not been demonstrated to result in specific adverse effects, but it has been considered as an important pathway for detoxification of

anticholinesterase compounds and has been often used as a surrogate measure of AChE inhibition [40].

Another example of a biomarker of effect is the micronucleus test. Micronuclei formation is a type of DNA alteration, which may result from chromosomal breakage (clastogenicity) or spindle anomalies [41]. The micronucleus test has been used to assess genotoxic effects on several bird species, namely in the herring gull (*Larus argentatus*) [42] and in the purple heron (*Ardea purpurea*), the little white egret (*Egretta garzetta*) and the cattle egret (*Bulbucus ibis*) [43].

- *Biomarker of susceptibility:* indicates an inherited or acquired ability of an organism to respond to the exposure of a specific toxic substance. Biomarkers of susceptibility may include alterations of genetic content, such as polymorphisms of activating systems and polymorphisms of detoxicating systems, and that may modify sensitivity of individuals to xenobiotics present in the environment [32].

An example of a biomarker of this type is paraoxonase (PON1). PON1 is an A-esterase present in serum and liver capable of hydrolyzing organophosphorous pesticides such as dioazinon and parathion, thus protecting against OP-poisoning [44]. PON1 activity varies greatly amongst animal species, and increased susceptibility to OP-poisoning is often related to decreased levels of this enzyme [44]. Birds, that have very low levels of this enzyme, thus being less effective in the detoxification of these compounds, are therefore particularly susceptible to OP-poisoning [44,45].

The use of biomarkers use on environmental risk assessment has been increasing progressively over the past years because they are believed to be among the most sensitive and earliest detectable responses in organisms [46]. Other examples of biomarkers that have been used to study wild populations of birds are avian eggshell thinning and induction of cytochrome P450. Eggshell thinning, which is caused by chlorinated hydrocarbons such as DDT, is a marker of reproductive impairment [32,39] and has been applied to numerous species such as the African darter (*Anhinga rufa*) [47] and the cattle egret (*Bulbucus ibis*) [48]. Cytochrome P450 is a family of enzymes involved in the metabolism of xenobiotic and endogenous substances and acts by

affecting their chemical structures [49]. Induction of cytochrome P450 has been used to diagnose organism's exposure to chemicals or substrates [49], and has been applied to several avian species including the black-crowned night-heron (*Nycticorax nycticorax*) [50], common cormorant (*Phalacrocorax carbo*) [51] and black-footed albatrosses (*Phoebastria nigripes*) [52].

1.2. Objectives and species studied

The main goals of this study were to answer the following questions:

1. Are some Portuguese populations of birds exposed to environmental contamination?

2. Could these factors lead to birds' illness and influence their recovery in wildlife rehabilitation centres?

3. Can ecotoxicological tools help to monitor and aid birds' recovery?

In order to address these issues, we measured selected enzymatic activities and assessed genotoxic exposure in aquatic wild birds from the orders Ciconiiformes, Charadriiformes and Pelecaniformes (Table 1).

All samples used were taken from a Portuguese wildlife rehabilitation centre (CERVAS-Centro de Ecologia, Recuperação e Vigilância de Animais Selvagens) and a nature reserve (PBG – Parque Biológico de Gaia). Due to the fact that all the samples obtained were dependent on the availability of animals and theirs residence in the rehabilitation centres, not all species were addressed in the three studies conducted. Detailed information on the species used for each study are given on Table 1 and on each following chapter.

Regarding the nature of the samples used, all samples taken from deceased animals (e.g. liver and brain samples) and live animals (e.g. blood) were generously provided by CERVAS and PBG. All procedures involving live bird handling were conducted accordingly to the Guide for the Care and Use of Laboratory Animals of the European Union, which is represented in Portugal by Decreto de Lei nº. 129/92 de 06 de Julho, Portaria nº. 1005/92 de 23 de Outubro de 1992.

Table 1.1. Overview of the species studied, with summarized information of the Order and Family characteristics. Adapted from [53-57].

Order	Family	Species
Ciconiiformes Long-legged and long-necked wading birds, their foot is broad but usually not webbed; the area between the eye and the base of the bill is usually devoid of feathers.	**Ardeidae** Medium-sized to large birds; mostly distributed on tropics, they are spread all over the world; normally feed in aquatic prey; migratory.	*Ardea cinerea* *Ardea purpurea* *Egretta garzetta*
	Ciconia Medium-sized to large birds with a cosmopolitan distribution; typical habitats include various kinds of wetlands and feed in small fish, frogs, insects and rodents.	*Ciconia ciconia*
Charadriiformes Diverse group of shore birds. Includes gulls, auks, plovers, etc.	**Laridae** Medium to large sized birds, they're typically coastal or inland species, rarely venturing far out to sea; ground nesting carnivores, the majority of the species will take live food or scavenge opportunistically; live prey include generally small fish and crabs.	*Larus michahellis*
Pelecaniformes Totipalmate swimmers with four toes included in the webbed foot; some might develop a throat pouch.	**Sulidae** Medium to large-sized, robust and cigar-shaped body birds; well adapted to living at sea, they plunge dive from great heights to capture fish; only *M. bassanus* and two other gannet species are truthfully migratory.	*Morus bassanus*

1.3. Relevance of the study

Aquatic ecosystems such as wetlands and coastal lagoons are highly productive environments that provide a wide range of ecosystem services. Nonetheless, due their limited ability to recover from disturbance, these habitats are particularly vulnerable to pollution which leads to environmental degradation with consequent inability to perform ecological services and ecosystem impairment [58].

Biomonitorization studies, such as the ones that aim to relate physicochemical analysis with the effects observed in the biological systems, play an important role in the assessment of contaminants' effects upon ecosystems [59]. A significant part of Portuguese biomonitoring studies has focused on organisms at lower trophic levels such as larvae, crustacean and mollusks, but failed to address contaminants' effects upon organisms at higher trophic levels such as mammals or birds.

Birds, including birds accepted in wildlife recovery units, reunite a multiple array of characteristics that makes them ideal indicators in environmental risk assessment studies. Functionally relevant components of ecosystems and highly sensitive towards environmental contaminants [27,30], studying birds allows an easy and effective way to evaluate effects of bioaccumulative contaminants and toxins at higher trophic levels.

Using birds accepted for rehabilitation at wildlife recovery centres does not only enables to screen for temporal and spatial trends of contamination in Portuguese populations of wild birds, but it also enables to assess whether background contamination could be related with their illness and at what extent it could impair bird recuperation. Moreover, using ecotoxicological tools to monitor birds during rehabilitation could be a valuable approach to help in birds' recovering. Thus, in a world with an increasing concern over environmental contamination and its role upon the actual decline of biodiversity, this type of approaches may play a crucial role in the conservation, rehabilitation and management of threatened bird species.

1.4. Organization of the thesis

The present thesis is organized into five chapters. The first one is the present "Introduction", the second, third and fourth chapters are structured as scientific papers and describe and discuss the results obtained. By order and contents, these are:

Chapter 1 - General Introduction, Objectives and Relevance of the work

Introductory chapter, describing wildlife toxicology and how this field emerged; the role of birds as bioindicator species and historical case studies of contaminant effects upon bird populations; lastly the main aims and relevance of the study are depicted.

Chapter 2 - Characterization of Cholinesterases in Plasma of Three Portuguese Native Bird Species: Application to Biomonitoring

Chapter describing plasma characterization of cholinesterases of three native species: the white stork (*Ciconia ciconia*), the grey heron (*Ardea cinerea*) and the northern gannet (*Morus bassanus*). In this chapter pseudocholinesterase is referred as butyrylcholinesterase.

Chapter 3 - Brain Cholinesterase Reactivation as a Marker of Pesticide Exposure in the Yellow-legged Gull *Larus michahellis* (Naumann, 1840): A Case Study.

Chapter describing the application of chemical and spontaneous reactivation assays in brain cholinesterase of the yellow-legged gull to investigate a possible case of pesticide poisoning.

Chapter 4 - Ciconiiformes as Sentinels of Chemicals' Genotoxic Effects: A 5-year Study on Portuguese Birds.

Chapter describing the evaluation of genotoxic damage in Ciconiiformes using the micronucleus test and the erythrocyte nuclear abnormalities (ENA) Assay.

Chapter 5: General Discussion and Conclusions

Provides a general discussion and conclusions of this study.

1.5. References

1. Wijbenga A, Hutzinger O (1984) Chemicals, man and the environment. Naturwissenschaften 71: 239-246.

2. Leblanc GA (2004) Basics of Environmental Toxicology. In: Hodgson E, editor. A Textbook of Modern Toxicology. New Jersey: John Wiley & Sons, Inc. pp. 463-467.

3. Hoffman D, Rattner B, Burton GA, Cairns J (2003) Handbook of Ecotoxicology; Hoffman D, Rattner B, Burton GA, Cairns J, editors. Boca Raton (FL): Lewis Publishers.

4. Fox GA (2000) Perturbations in terrestrial vertebrate populations: Contaminants as a cause. Environmental Contaminants and Terrestrial Vertebrates: Effects on Populations, Communities, and Ecosystems: 19-59.

5. Kendall RJ (2010) Introduction and Overview. In: Kendall RJ, Lacher TE, Cobb GP, Cox SB, editors. Toxicology: Emerging Contaminantsand Biodiversity Issues. Boca Raton: CRC Press.

6. Rattner BA (2009) History of wildlife toxicology. Ecotoxicology 18: 773-783.

7. Pattee OH, Pain DJ (2003) Lead in the Environment. In: Hoffman DJ, Rattner BA, Burton GA, Cairns J, editors. Handbook of Ecotoxicology. Second ed. Boca Raton (FL): Lewis Publishers. pp. 373-408.

8. Alberts PH (2003) Petroleum and Individual Polycyclic Aromatic Hydrocarbons. In: Hoffman DJ, Rattner BA, Burton GA, Cairns J, editors. Handbook of Ecotoxicology. Second ed. Boca Raton (FL): Lewis Publishers. pp. 341-371.

9. Coburn DR, Dewitt JB, Derby JV, Ediger E (1950) Phosphorus Poisoning in Waterfowl. Journal of the American Pharmaceutical Association-Scientific Edition 39: 151-158.

10. Sotherton N, Holland J (2003) Indirect effects of pesticides on farmland wildlife. In: Hoffman DJ, Rattner BA, Burton GA, Cairns J, editors. Handbook of Ecotoxicology. Second ed. Boca Raton (FL): Lewis Publishers. pp. 373-408.

11. Fossi MC, Massi A, Leonzio Ceg (1994) Blood esterase inhibition in birds as an index of organophosphorus contamination: field and laboratory studies. Ecotoxicology: 11-20.

12. Smith PN, Cobb GP, Godard-Codding C, Hoff D, McMurry ST, et al. (2007) Contaminant exposure in terrestrial vertebrates. Environmental Pollution 150: 41-64.

13. Sheffield SR, Sullivan JP, Hill EF (2012) Identifying and Handling Contaminant-Related Wildlife Mortality or Morbidiy. Volume 1: Research. In: Silvy NJ, editor. The Wildlife Thecniques Manual Research 7th ed. Baltimore: Johns Hopkins University Press. pp. 154-180.

14. Parsons KC, Matz AC, Hooper MJ, Pokras MAeg (2000) Monitoring Wading Bird Exposure to Agricultural Chemicals Using Serum Cholinesterase Activity. Environmental Toxicology and Chemistry 19.

15. Koster MD, Ryckman DP, Weseloh DVC, Struger J (1996) Mercury levels in great lakes herring gull (Larus argentatus) eggs, 1972-1992. Environmental Pollution 93: 261-270.

16. Poppenga RH (2007) Avian toxicology. In: C. GR, editor. Veterinary Toxicology: Basic and Clinical Principles. USA: Academic Press. pp. 663-688.

17. Lundholm CE (1997) DDE-induced eggshell thinning in birds: Effects of p,p'-DDE on the calcium and prostaglandin metabolism of the eggshell gland. Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology 118: 113-128.

18. Henny CJ, Grove RA, Kaiser JL, Johnson BL (2010) North American Osprey Populations and Contaminants: Historic and Contemporary Perspectives. Journal of Toxicology and Environmental Health-Part B-Critical Reviews 13: 579-603.

19. Grasman KA, Scanlon PF, Fox GA (1998) Reproductive and physiological effects of environmental contaminants in fish-eating birds of the Great Lakes: A review of historical trends. Environmental Monitoring and Assessment 53: 117-145.

20. Ratcliff.Da (1967) Decrease in Eggshell Weight in Certain Birds of Prey. Nature 215: 208-&.

21. Hickey JJ, Anderson DW (1968) Chlorinated Hydrocarbons and Eggshell Changes in Raptorial and Fish-Eating Birds. Science 162: 271-&.

22. Soler F, Oropesa AL, Perez-Lopez M, Hernandez D, Garcia JP, et al. (2007) Acetylcholinesterase activity in seabirds affected by the Prestige oil spill on the Galician coast (NW Spain). Science of The Total Environment 372: 532-538.

23. Grau CR, Roudybush T, Dobbs J, Wathen J (1977) Altered Yolk Structure and Reduced Hatchability of Eggs from Birds Fed Single Doses of Petroleum Oils. Science 195: 779-781.

24. Fry DM, Swenson J, Addiego LA, Grau CR, Kang A (1986) Reduced Reproduction of Wedge-Tailed Shearwaters Exposed to Weathered Santa-Barbara Crude-Oil. Archives of Environmental Contamination and Toxicology 15: 453-463.

25. Karr JR (2008) Ecological Health Indicators. In: Jorgensen SE, Fath BD, editors. Encyclopedia of Ecology. Amsterdam: Elsevier. pp. 1037-1041.

26. Holt EA, Miller SW (2011) Bioindicators: Using Organisms to Measure Environmental Impacts. Nature Education Knowledge 2: 8.

27. Mistry J, Berardi A, Simpson M (2008) Birds as indicators of wetland status and change in the North Rupununi, Guyana. Biodiversity and Conservation 17: 2383-2409.

28. Goldstein MI, Lacher TE, Zaccagnini ME, Parker ML, Hooper MJ (1999) Monitoring and Assessment of Swainson's Hawks in Argentina Following Restrictions on Monocrotophos Use, 1996–97. Ecotoxicology 8: 215-224.

29. Monteiro LR, Furness RW (1995) Seabirds as monitors of mercury in the marine environment. Water, Air, & Soil Pollution: 851-870.

30. Stolen ED, Breininger DR, Frederick PC (2005) Using waterbirds as indicators in estuarine systems: successes and perils. Estuarine Indicators: 409-422.

31. WHO/IPCS (1993) Biomarkers and Risk Assessment: concepts and principles. Environmental Health Criteria 155. Geneva: World Health Organization.

32. Gil F, Pla A (2001) Biomarkers as biological indicators of xenobiotic exposure. Journal of Applied Toxicology 21: 245-255.

33. Tavares PC, Monteiro LR, Lopes RJ, Correia Santos MM, Furness RW (2004) Intraspecific Variation of Mercury Contamination in Chicks of Black-Winged Stilt (Himantopus himantopus) in Coastal Wetlands from Southwestern Europe. Bulletin of Environmental Contamination and Toxicology 72: 437-444.

34. García-Hernández J, Sapozhnikova YV, Schlenk D, Mason AZ, Hinojosa-Huerta O, et al. (2006) Concentration of contaminants in breeding bird eggs from the Colorado River delta, Mexico. Environmental Toxicology and Chemistry 25: 1640-1647.

35. Radic Z, Taylor P (2006) Structure and Function of Cholinesterases. In: Gupta RC, editor. Toxicology of Organophosphate and Carbamate Pesticides. Waltham: Elsevier Academic Press. pp. 161-186.

36. Fleming WJ (1981) Recovery of Brain and Plasma Cholinesterase Activities in Ducklings Exposed to Organo-Phosphorus Pesticides. Archives of Environmental Contamination and Toxicology 10: 215-229.

37. Hunt KA, Hooper MJ, Littrell EE (1995) Carbofuran Poisoning in Herons - Diagnosis Using Cholinesterase Reactivation Techniques. Journal of Wildlife Diseases 31: 186-192.

38. Goldstein MI, Lacher TE, Woodbridge B, Bechard MJ, Canavelli SB, et al. (1999) Monocrotophos-induced mass mortality of Swainson's hawks in Argentina, 1995-96. Ecotoxicology 8: 201-214.

39. Mayer FL, Versteeg DJ, McKee MJ, Folmar LC, Graney RL, et al. (1992) Physiological and nonspecific biomarkers. In: Huggett RJ, Kimerie RA, Mehrle PMJ, Bergman HL, editors. Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropohenic Stress. Boca Raton: Lewis Publishers. pp. 5-85.

40. Sultatos LG (2006) Interactions of organophosphorus and carbamate compounds with cholinesterases. In: Gupta RC, editor. Toxicology of Organophosphate and Carbamate Pesticides. Waltham: Elsevier Academic Press. pp. 209-218.

41. Shugart L, Bickham J, Jackim G, McMahon G, Ridley W, et al. (1992) DNA alterations. In: Huggett RJ, Kimerie RA, Mehrle PMJ, Bergman HL, editors. Biomarkers:

Biochemical, Physiological, and Histological Markers of Anthropohenic Stress. Boca Raton: Lewis Publishers. pp. 125-153.

42. Skarphedinsdottir H, Hallgrimsson GT, Hansson T, Hagerroth PA, Liewenborg B, et al. (2010) Genotoxicity in herring gulls (Larus argentatus) in Sweden and Iceland. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 702: 24-31.

43. Quiros L, Ruiz X, Sanpera C, Jover L, Pina B (2008) Analysis of micronucleated erythrocytes in heron nestlings from reference and impacted sites in the Ebro basin (NE Spain). Environmental Pollution 155: 81-87.

44. Costa LG, Richter RJ, Li WF, Cole T, Guizzetti M, et al. (2003) Paraoxonase (PON1) as a biomarker of susceptibility for organophosphate toxicity. Biomarkers 8: 1-12.

45. Li WF, Costa LG, Furlong CE (1993) Serum Paraoxonase Status - a Major Factor in Determining Resistance to Organophosphates. Journal of Toxicology and Environmental Health 40: 337-346.

46. Jemec A, Drobne D, Tisler T, Trebse P, Ros M, et al. (2007) The applicability of acetylcholinesterase and glutathione S-transferase in Daphnia magna toxicity test. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 144: 303-309.

47. Bouwman H, Polder A, Venter B, Skaare JU (2008) Organochlorine contaminants in cormorant, darter, egret, and ibis eggs from South Africa. Chemosphere 71: 227-241.

48. Malik RN, Rauf S, Mohammad A, Eqani S, Ahad K (2011) Organochlorine residual concentrations in cattle egret from the Punjab Province, Pakistan. Environmental Monitoring and Assessment 173: 325-341.

49. Stegeman JJ, Brouwer M, Di Giulio RT, Förlin L, Fowler BA, et al. (1992) Molecular responses to environmental contamination. In: Huggett RJ, Kimerie RA, Mehrle PMJ, Bergman HL, editors. Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress. Boca Raton: Lewis Publishers. pp. 125-153.

50. Rattner BA, Melancon MJ, Rice CP, Riley W, Eisemann J, et al. (1997) Cytochrome P450 and organochlorine contaminants in black-crowned night-herons from the Chesapeake Bay region, USA. Environmental Toxicology and Chemistry 16: 2315-2322.

51. Kubota A, Iwata H, Tanabe S, Yoneda K, Tobata S (2005) Hepatic CYP1A Induction by Dioxin-like Compounds, and Congener-Specific Metabolism and Sequestration in Wild Common Cormorants from Lake Biwa, Japan. Environmental Science & Technology 39: 3611-3619.

52. Kubota A, Watanabe M, Kunisue T, Kim EY, Tanabe S, et al. (2010) Hepatic CYP1A Induction by Chlorinated Dioxins and Related Compounds in the Endangered Black-Footed Albatross from the North Pacific. Environmental Science & Technology 44: 3559-3565.

53. Hoyo J, Elliot A, Sargatal J, others a (1992) Handbook of the Birds of the World. Volume 1- Ostrich to Ducks. Barcelona: Lynx Edicions.

54. Cramp S, Simmons KEL, Brooks DJ, Collar NJ, Dunn E, et al. (1978) Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palaearctic. ; Cramp S, editor. Oxford: Oxford University Press

55. Cramp S, Simmons KEL, Brooks DJ, Collar NJ, Dunn E, et al. (1983) Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palaearctic. ; Cramp S, editor. Oxford: Oxford University Press

56. Cramp S, Simmons KEL, Brooks DJ, Collar NJ, Dunn E, et al. (1985) Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palaearctic. ; Cramp S, editor. Oxford: Oxford University Press

57. Heinzel H, Fitter R, Parslow J (1998) The birds of Britain and Europe with North Africa and the Middle East London: Harper Collins.

58. Machado DA, Imberger J (2012) Managing wastewater effluent to enhance aquatic receiving ecosystem productivity: A coastal lagoon in Western Australia. Journal of Environmental Management 99: 52-60.

59. Monteiro MSSCA (2003) Avaliação da toxicidade de contaminantes ambientais em populações naturais de Pomatoschistus microps (Krøyer, 1938). Aveiro: University of Aveiro.

Chapter 2

Characterization of Cholinesterases in Plasma of Three Portuguese Native Bird Species: Application to Biomonitoring

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2. Characterization of Cholinesterases in Plasma of Three Portuguese Native Bird Species: Application to Biomonitoring

2.1 Abstract

Over the last decades the inhibition of plasma cholinesterase (ChE) activity has been widely used as a biomarker to diagnose organophosphate and carbamate exposure. Plasma ChE activity is a useful and non-invasive method to monitor bird exposure to anticholinesterase compounds; nonetheless several studies had shown that the ChE form(s) present in avian plasma may vary greatly among species. In order to support further biomonitoring studies and provide reference data for wildlife risk-assessment, plasma cholinesterase of the northern gannet (*Morus bassanus*), the white stork (*Ciconia ciconia*) and the grey heron (*Ardea cinerea*) were characterized using three substrates (acetylthiocholine iodide, propionylthiocholine iodide, and S-butyrylthiocholine iodide) and three ChE inhibitors (eserine sulphate, BW284C51, and iso-OMPA). Additionally, the range of ChE activity that may be considered as basal levels for non-exposed individuals was determined. The results suggest that in the plasma of the three species studied the main cholinesterase form present is butyrylcholinesterase (BChE). Plasma BChE activity in non-exposed individuals was 0.48±0.11 SD U/ml, 0.39±0.12 SD U/ml, 0.15±0.04 SD U/ml in the northern gannet, white stork and grey heron, respectively. These results are crucial for the further use of plasma BChE activity in these bird species as a contamination bioindicator of anti-cholinesterase agents in both wetland and marine environments. Our findings also underscore the importance of plasma ChE characterization before its use as a biomarker in biomonitoring studies with birds.

Keywords: Biomarkers, butyrylcholinesterase, *Ardea cinerea*, *Ciconia ciconia, Morus bassanus*.

2.2 Introduction

Cholinesterase (ChE) activity has been routinely used as a biomarker to diagnose exposure to anticholinesterase compounds such as organophosphate (OP) and carbamate (CB) pesticides. These pesticides are broadly used to control insect pests and disease vectors; nonetheless, they can be extremely toxic to non-target organisms like mammals and birds [1,2,3,4]. They act by inhibiting the activity of cholinesterases, which causes an over accumulation of acetylcholine at the synapses and consequent disruption of nerve function, leading to subsequent physiologic disorders and ultimately death [5]. In addition to OPs and CBs, other environmental contaminants such as metals, detergents and petroleum-derived products have been found to generate similar inhibitory effects [6,7,8]. Serum or plasma has been broadly used to measure ChE activity as a non-invasive method to monitor exposure of wildlife to pesticides in the field due to its sensitivity to ChE-inhibiting compounds [9,10,11,12]. Nonetheless, its use requires the characterization of the enzyme form(s) present in the tissue assayed and the determination of the normal range of activity in non-exposed individuals [13].

Two enzymes form the family of cholinesterases: acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8). Both catalyze the hydrolysis of the neurotransmitter acetylcholine, but differ in substrate specificity and inhibitor susceptibility [14,15]; tissue distribution can also vary, depending on the organism measured. AChE is predominantly found in the neuromuscular junctions and central nervous system, playing a key-role in the cholinergic neurotransmission, while BChE is mainly found in serum and liver, but its primary physiological role remains unknown [14].

Regarding wildlife exposure to environmental contaminants, waterbirds, such as wading birds and seabirds, are useful indicators of environmental variation upon short and long temporal scales [16]. Wading birds are primarily indicators of wetland quality as they can occupy a wide variety of foraging niches, including agricultural ponds, which makes them often non-target species to OP and CB exposure through ingestion and dermal contact [12]. In the case of seabirds, they are widely used to monitor the occurrence and ecological impacts of contaminants such as oil and mercury in the marine environment [17]. All these possible exposures may lead into an impairment of ChE activities in birds, and therefore biomarkers like this may be also a useful indicator to detect contamination in birds' habitats. In birds, AChE is found in the brain while BChE is mainly present in plasma; nonetheless, several studies had shown that AChE and other esterases (e.g.

carboxylesterase- CbE) might also occur in avian plasma with wide interspecies differences [3,18].

In order to use plasma ChE as a biomarker of exposure in three Portuguese native bird species, the main aim of this study was to: (i) characterize the ChE form(s) present in birds' plasma, (ii) determine the basal levels of ChE activity in non-exposed individuals and (iii) establish the appropriate assay conditions for the use of plasma ChE activity, using as bird species: the grey heron (*Ardea cinerea*) and the white stork (*Ciconia ciconia*), two wading bird species resident in Portugal, and the northern gannet (*Morus bassanus*), a migratory seabird common along the Portuguese coast during winter.

2.3 Materials and methods

2.3.1 Sample collection

All the species of birds used to characterize plasma ChE were adult individuals inhabiting the Gaia Biological Park, a nature reserve located in Avintes (Porto, Portugal). *C. ciconia* individuals were free-living in the park while individuals of *A. cinerea* and *M. Bassanus* were in captivity. The disturbance stress caused by the animal handling was minimized by limiting the visit length, avoiding any sampling during extreme weather conditions (e.g. heavy rain, low temperatures) and using a small mantle to cover the head.

Blood was drawn from the brachial vein with sterile 1-ml syringes and 25-ga needles, and it was collected into a capillary tube with EDTA (Microvette® CB 300, Sarstedt). Following centrifugation, plasma was extracted and stored at -80ºC until analysis.

2.3.2 Sample preparation and ChE determinations

Plasma samples were diluted in phosphate buffer (0.1M, pH 7.2), and ChE activity was determined in quadruplicate according to the Ellman method [19] adapted to microplate [20] using a microplate reader (Thermo Scientific Multiskan® Spectrum). For all species, plasma dilutions for each individual were prepared using 2µl of plasma (2-µl micropipette, Gilson®) for a final assay volume of 1 ml. The enzymatic activity was expressed in units (U) per ml of plasma (1U is a µmol of substrate hydrolyzed per minute).

2.3.3 Cholinesterase characterization

Plasma ChE was characterized by testing the substrate preferences of the enzymes and their sensitivity to selective inhibitors. In independent experiments, acetylthiocholine iodide (AcSCh), S-butyrylthiocholine iodide (BuSCh) and propionylthiocholine iodide (PrSCh) were used as substrates at increasing concentrations (from 0.005 to 20.5mM) and the enzymatic activity was determined.

Eserine sulphate, 1,5-bis(4-allyldimethyl-ammonimphenyl)pentan-3-one dibromide (BW284C51) and tetraisopropyl pyrophosphoramide (iso-OMPA) were selected as selective inhibitors of all ChE(s), AChE and BChE, respectively [21,22]. For each inhibitor, stock solutions were prepared in ultrapure water or ethanol, as appropriate, with concentrations ranging from 6.25 to 200 µM (eserine and BW284C51) and from 0.25 to 8.0 mM (iso-OMPA). For each inhibitor, 5 µl of a stock solution was incubated with 495 µl of the sample during 30 min, at 25 ±1ºC, before substrate addition. ChE was then assayed using both AcSCh and BuSCh as substrates. Ultrapure water was added to the controls and an additional control with ethanol was used in the experiments with iso-OMPA. In all characterization procedures, three samples of plasma corresponding to three adults per specie were used.

2.3.4 Basal level for the activity of the dominant ChE

In order to determine the normal activity range of the dominant form of ChE present in the plasma of *M. bassanus*, *C. ciconia* and *A. cinerea*, samples of plasma from non-exposed individuals were assayed as previously described and the appropriated substrate was used. In this case, as BChE was the dominant form present (see details in the Results section) BuSCh was used as substrate at a concentration of 10.24 mM. The number of individuals ranged from 4-5 per bird species; sub-replicates of each individual ranged from 8-16.

2.3.5 Chemicals

5,5'-Dithiobis(2-nitrobenzoic acid), AcSCh, BuSCh, PrSCh, eserine hemisulphate, iso-OMPA, and BW284C51 were obtained from Sigma-Aldrich Europe (Netherlands). All the other chemicals used in this experiment were purchased from Merck (Germany).

2.3.6 Data analysis

Analysis of variance (ANOVA) was performed to compare differences between inhibitor concentrations when the criteria of normality and equality of variance were satisfied (whenever necessary, data were transformed using $\log_{10}$ or square root). Dunnet's test was used to discriminate statistical differences between treatments and the control. All data analyses were performed using SigmaStat® 3.5 software (Systat Software Inc.).

2.3.7 Ethics statement

All procedures involving bird handling were conducted according to the Guide for the Care and Use of Laboratory Animals of the European Union - in Portugal represented by Decreto de Lei nº 129/92 de 06 de Julho, Portaria nº 1005/92 de 23 de Outubro de 1992. Approval by a named review board institution or ethics committee was not necessary as the final model for ethical experimentation using animals is yet to be implemented in Portuguese research units.

2.4 Results

In order to investigate substrate preferences of ChE in plasma of the species studied, the substrates AcSCh, PrSCh and BuSCh were assayed at increasing concentrations (Fig. 2.1). Maximum enzyme activity in *M. bassanus* was observed with AcSCh at 10.24 mM (1.14±0.08 SE U/ml), while in *C. ciconia* and *A. cinerea* maximum activity was obtained with PrSCh at 20.48 mM (0.97±0.11 SE U/ml) and 5.15 mM (0.32±0.03 SE U/ml), respectively. The enzyme kinetic parameters V_{max} (maximum rate of hydrolysis reached when the enzyme is saturated with substrate) and K_m (concentration needed to reach one-half of the maximum velocity) for the species studied are depicted on Table 2.1. Highest enzyme affinity in *M. bassanus* was obtained with AcSCh (K_m=15.3 µM), while in *C. ciconia* was obtained with BuSCh (K_m= 4.8 µM) and *A. cinerea* with PrSCh (K_m= 150.1 µM).

Several works have indicated that most of the total ChE activity in plasma of different bird species is attributable to BChE [e.g. 23, 24]; therefore all inhibitor assays were performed using BuSCh, cleaved preferentially by BChE, and also using AcSCh, cleaved preferentially by AChE, both at the concentration of 10.24 mM. This allowed a better differentiation of the ChE form(s) present.

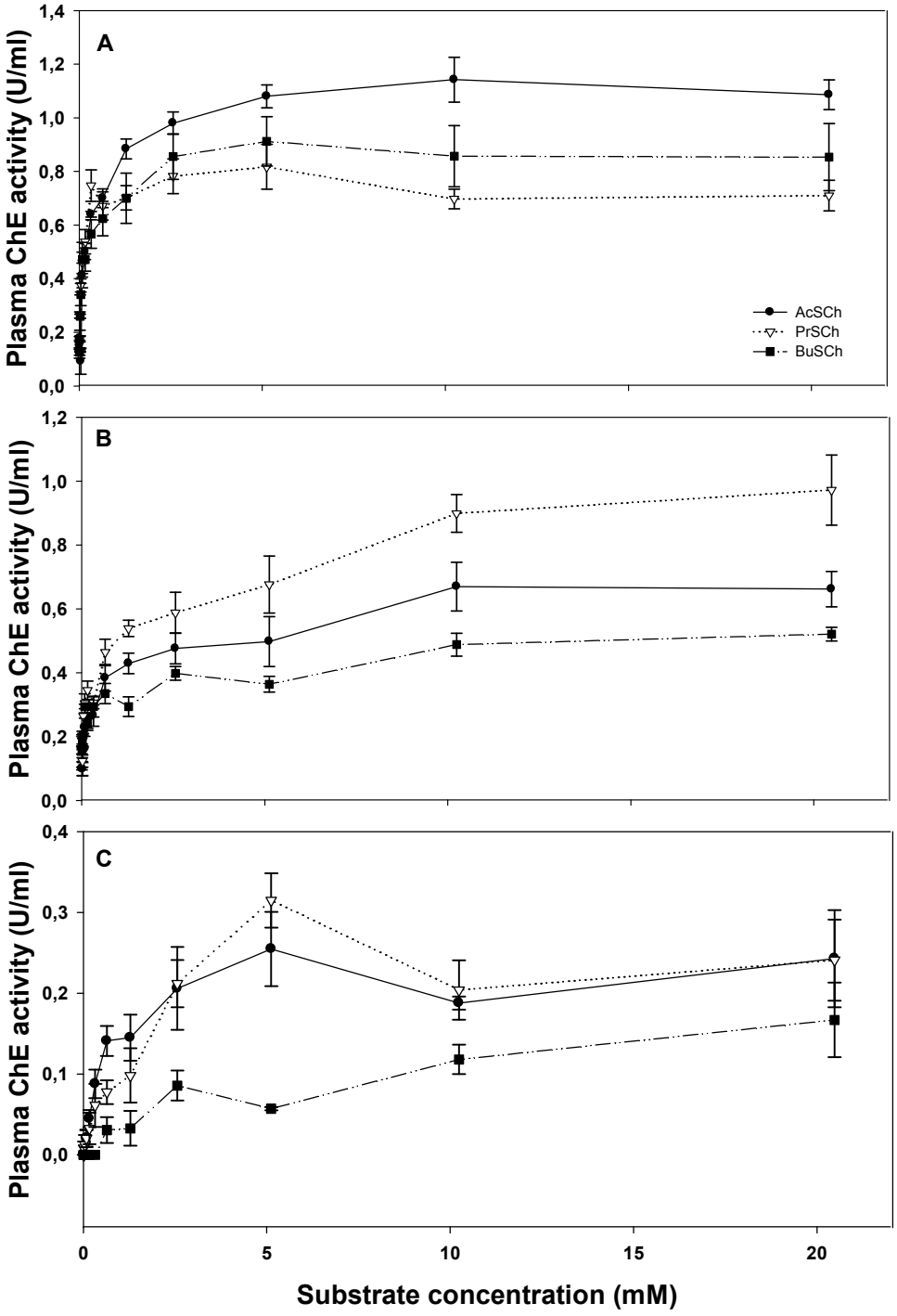

Fig. 2.1. Plasma ChE activity at increasing concentrations of the substrates acetylthiocholine iodide (AcSCh), iodide propionylthiocholine iodide (PrSCh) and *S*-butyrylthiocholine iodide (BuSCh) in: (A) *M. bassanus*, (B) *C. ciconia* and (C) *A. cinerea*. Results are expressed as the mean ± SE of three birds.

The effects of the inhibitor eserine sulphate are presented in Fig. 2.2. A clear inhibition of ChE activity ($P<0.05$) was observed in all species studied at the lowest inhibitor concentration using both substrates, except for *A. cinerea* (Fig. 2.2 C) at 6.25-25 μM of

eserine, when using BuSCh as a substrate. Inhibitions at 200 μM of eserine were about 56% and 91% in *M. bassanus*, 90% and 100% in *C. ciconia*, and 98% and 100% in *A. cinerea*, using BuSCh and AcSCh respectively. No effects on the ChE activity of the three species were reported when the selective inhibitor for AChE, BW284C51, was assayed at concentrations up to 200 μM ($P<0.05$), using both substrates (Fig. 2.2).

In all species iso-OMPA, which is the selective inhibitor of BChE, significantly inhibited ChE activity in the presence of the substrates BuSCh and AcSCh ($P<0.05$) (Fig. 2.3). Inhibitions of about 82% with BuSCh and 100% with AcSCh in *M. bassanus* (Fig. 2.3-A) were obtained at 8 mM of inhibitor. At the same concentration of iso-OMPA, inhibitions of about 95% and 97% were observed in *C. ciconia* (Fig. 2.3-B), using BuSCh and AcSCh respectively. In *A. cinerea*, inhibitions of about 88% and 69% were observed with 8 mM of iso-OMPA, using BuSCh and AcSCh respectively.

The basal levels of BChE activity in plasma for the three species are depicted on Table 2.2. The variation in mean plasma BChE activity across species was 0.48±0.11 U/ml in *M. bassanus*, 0.39±0.12 U/ml in *C. ciconia* and 0.15±0.04 U/ml in *A. cinerea*.

Table 2.1. Apparent values of K_m(μM) and V_{max}(μmol/min/min) as estimated by Michaelis-Menten equation for the substrates AcSCh, BuSCh and PrSCh. Values for this study are expressed as the mean value of 3 individuals.

Species	AcSCh		BuSCh		PrSCh	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
M. bassanus	15.3	0.59	19.4	0.56	21.4	0.64
C. ciconia	10.7	0.27	4.8	0.29	6.1	0.34
A. cinerea	246.8	0.15	166.8	0.08	150.1	0.12

2.5 Discussion

The first aim of this study was to characterize the ChE form(s) present in plasma of *M. bassanus*, *C. ciconia* and *A. cinerea*. In order to achieve that, the first step was to distinguish ChE form(s) from nonspecific esterases. This procedure is essential to avoid potential sources of error in biomarker studies because tissues may contain significant amounts of nonspecific esterases that might contribute to the total enzymatic activity measured, but differ in their sensitivities towards ChE-inhibiting compounds [13]. The presence of nonspecific esterases was determined using eserine sulphate, which is a

selective inhibitor of all cholinesterase activity in the range of 10^{-6} to 10^{-5} M [21]. In this study, the enzymatic activity measured in plasma of the studied species was almost completely inhibited by eserine within the μM range, which indicates that the predominant enzyme(s) present is (are) ChE(s) and not other type of esterases.

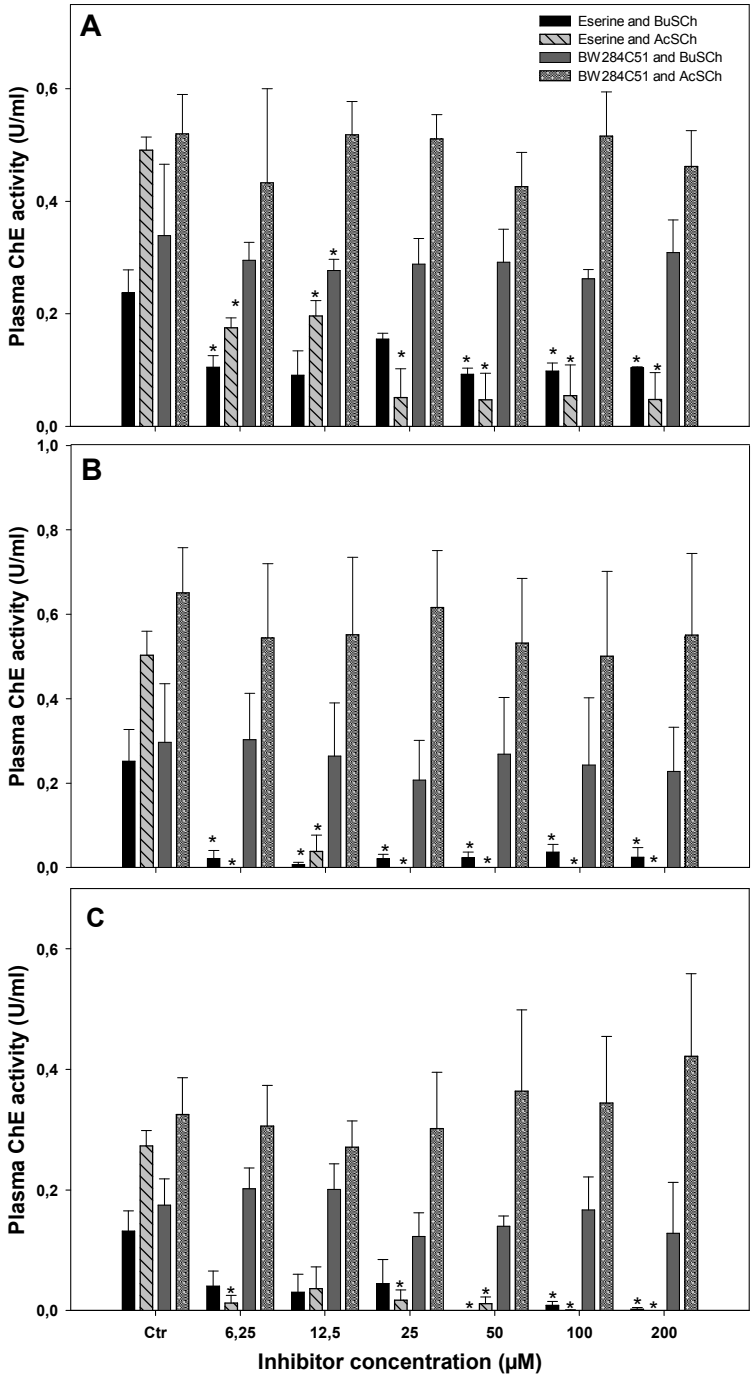

Fig. 2.2. Effects of the inhibitors eserine and BW284C51 on the plasma ChE activity of: (A) *M. bassanus* , (B) *C. ciconia* and (C) *A. cinerea* using AcSCh or BuSCh as substrates. Results are expressed as the mean ± SE of three birds; *significantly different from control (*P<0.*05).

In order to further distinguish which ChE form(s) was (were) present, the substrate preference of the enzyme and its sensitivity to BW284C51 and iso-OMPA was tested. ChE activity measured in plasma of *M. bassanus* showed a preference for AcSCh (Fig. 1A); in contrast, plasma ChE of *C. ciconia* and *A. cinerea* displayed a substrate preference towards PrSCh (Figs 1B and 1C). Also, enzymatic activity was observed to stabilize at higher substrate concentrations, with no decrease of ChE activity. The higher rate of enzymatic activity registered with AcSCh in *M. bassanus* could be interpreted as a sign of the presence of AChE, although enzyme inhibition at higher substrate concentrations wasn't observed as it would be expected for a typical vertebrate AChE; additionally, a relatively high enzyme activity was obtained when using BuSCh (about 76% of the activity obtained with AcSCh at 5.12 mM), which is considered a specific substrate for BChE [23]. The other two bird species showed a preference towards PrSCh, but even though BuSCh was the substrate cleaved at the lowest rate, enzyme activity with this substrate was considerably high (about 54% and 78% of the activity obtained with PrSCh at 20.5 mM, in *C. ciconia* and *A. cinerea,* respectively). These results seem to indicate the presence of both ChE forms, AChE and BChE. This is in accordance with numerous studies which have indicated that most of the total ChE activity in plasma of birds is attributable to BChE, even though AChE activity may also occur at significant rates and the ratio between the two ChE forms may vary greatly among species [3,18,24]. After this first approach, to better characterize the ChE(s) present in plasma of the species studied, the inhibition assays with BW284C51 and iso-OMPA were performed using both AcSCh and BuSCh, in order to clarify which would be the most dominant form.

Table 2.2. Normal range of ChE activity in non-exposed individuals of the Northern Gannet, the White stork and the Grey heron respectively, including sample size of individuals (n), minimum (min), maximum (max), mean and standard deviation (SD) values.

Species	**BChE Activity (U/ml)**				
	n	**Min**	**Max**	**Mean**	**SD**
M. bassanus	5	0.36	0.64	0.48	0.107
C. ciconia	5	0.26	0.54	0.39	0.124
A. cinerea	4	0.10	0.21	0.15	0.044

In all species studied, no effect on ChE activity was observed with BW284C51, selective inhibitor of AChE, using both AcSCh and BuSCh; furthermore, plasma ChE activity was strongly inhibited by iso-OMPA using both AcSCh and BuSCh. This seems to

indicate the presence in all species of an enzyme that cleaves both AcSCh and BuSCh, that is resistant to BW284C51 (an AChE inhibitor) but highly sensitive to iso-OMPA (a specific inhibitor of BChE activity) [22]. Therefore, these results suggest that the enzyme present in plasma of the studied species is BChE. These results are in accordance with the majority of the studies published in this research area for birds. For example, Strum et al. [3] and Fildes et al. [24] have also found BChE to be the predominant ChE form present in the plasma of the sparrowhawk (*Accipiter nisus*), the Egyptian vulture (*Neophron percnopterus*), the brown songlark (*Cincloramphus cruralis*) and the australasian pipit (*Anthus novaeseelandiae*).

Like other vertebrates, in the present study plasma BChE activity of the northern gannet (*M. bassanus*), the white stork (*C. ciconia*) and the grey heron (*A. cinerea*) showed a Michaellis-Menten behaviour. Plasma BChE of *M. bassanus* and *C. ciconia* showed a higher affinity to BuSCh (K_m=19.4 and 4.8 μM, respectively) than other vertebrates such as the mink *Mustela vison (*K_m=240 μM) and the lizard *Gallotia galloti* (K_m=1x10^3 μM), but similar to the pigeon *Columbia livia* (K_m=32 μM) and the rat *Rattus novergicus* (K_m= 13 μM) [25,26]. The K_m values of the grey heron (*A. cinerea*) obtained with BuSCh was higher than the ones reported for the other two studied species, but smaller than the ones obtained with *M. vison*, *G. galloti* and the fish *Pioractus mesopotamicus* (K_m= 1.2x10^3 μM) [25,26].

Plasma BChE activity in non-exposed individuals of *M. bassanus*, *C. ciconia* and *A. cinerea* are within the range of values reported in the literature for several non-exposed birds. For example, mean BChE activity values measured for the kestrel (*Falco tinnunculus*), the buzzard (*Buteo buteo*) and the tawny owl (*Strix aluco*) were 0.28±0.08 SD U/ml, 0.91±0.8 SD U/ml and 1.49±0.36 SD U/ml, respectively [18]. Goldstein et al. [27] also measured a mean BChE activity of 0.41±0.09 SD U/ml in the swainson hawk (*Buteo swainsoni*), and Strum et al. [3] found BChE values of 0.83±0.01 SD U/ml in the American golden-plover (*Pluvialis dominica*) and 1.15±0.45 SD U/ml in the killdeer (*Charadrius vociferous*). The baseline plasma BChE values of the species presented in this study will provide a useful tool in the future for comparison with potential intoxicated/stressed birds but its use should be performed with caution. ChE activity may vary with several natural factors such as season, temperature and life stage [15]. Moreover, it should be taken into consideration that the sampling size of the obtained measurements is relatively low, even though a high number of replicates per individual were performed to decrease experimental error.

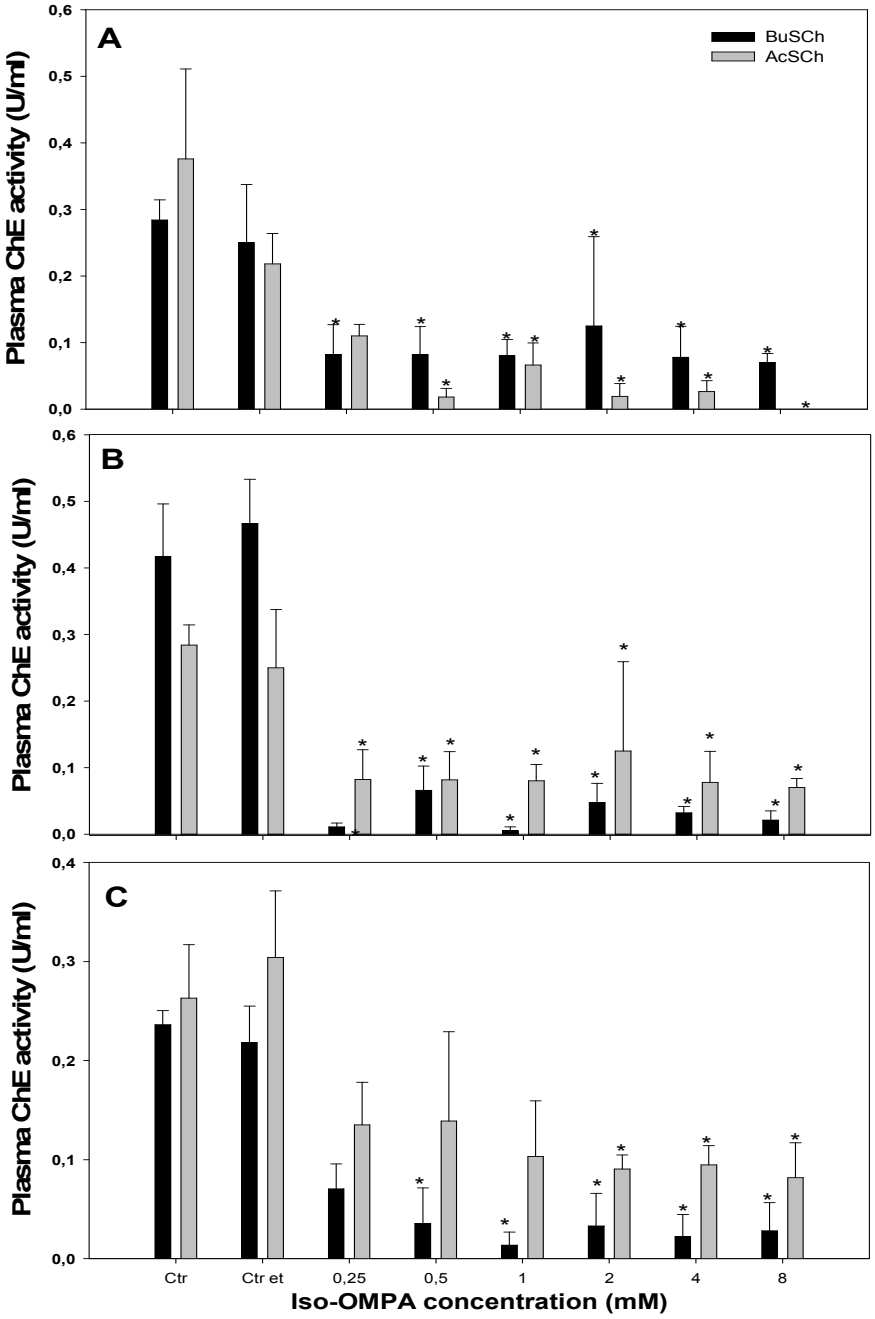

Fig. 2.3. Effects of the inhibitor iso-OMPA on the plasma ChE activity of: (A) *M. bassanus* , (B) *C. ciconia* and (C) *A. cinerea* using AcSCh or BuSCh as substrates. Results are expressed as the mean ± SE of three birds; *significantly different from control (*P<0.*05).

For future works with these species, it is recommendable the use of BuSCh at ~10 mM, as it is a specific substrate for BChE and because in the species studied the maximum enzyme activity with this substrate was observed to be stable at that concentration.

In conclusion, the variability in the ChE activity of the species studied compared to other avian species previously studied underscores the importance of plasma ChE characterization before its use as a biomarker in biomonitorization studies with birds. For further use of plasma ChE activity as biomarker to diagnose exposure to anticholinesterase compounds in *M. bassanus*, *C. ciconia* and *A. cinerea* , BuSCh seems to be the most suitable substrate for enzymatic measurements, since BChE was found to be the predominant ChE form present in the species studied. The data presented here provide a starting point for the use of plasma BChE activity as a biomarker in Portuguese native bird species, aiding field investigations and monitoring risk of exposure of non-target wildlife to ChE-inhibiting compounds. In addition, the measurement of BChE activity may be a promising tool to be used in birds kept in captivity and recovering from physical or chemical stress, to evaluate their fitness and possible release.

2.6. Acknowledgements

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2.7. References

1. Hill EF (2003) Wildlife Toxicology of Organophosphorus and Carbamate Pesticides. In: Hoffman DJ, Rattner BA, Burton GA, Cairns J, editors. Handbook of Ecotoxicology. Second ed. Boca Raton: Lewis Publishers. pp. 281-312.
2. Blakley BR, Yole MJ (2002) Species differences in normal brain cholinesterase activities of animals and birds. Veterinary and Human Toxicology 44: 129-132.
3. Strum KM, Alfaro M, Haase B, Hooper MJ, Johnson KA, et al. (2008) Plasma cholinesterases for monitoring pesticide exposure in Nearctic-Neotropical migratory shorebirds. Ornitologia Neotropical 19: 641-651.
4. Grue CE, Gibert PL, Seeley ME (1997) Neurophysiological and behavioral changes in non-target wildlife exposed to organophosphate and carbamate pesticides: Thermoregulation, food consumption, and reproduction. American Zoologist 37: 369-388.
5. Franson JC, Smith MR (1999) Poisoning of wild birds from exposure to anticholinesterase compounds and lead: Diagnostic methods and selected cases. Seminars in Avian and Exotic Pet Medicine 8: 3-11.
6. Frasco MF, Fournier D, Carvalho F, Guilhermino L (2005) Do metals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. Biomarkers 10: 360-375.
7. Guilhermino L, Barros P, Silva MC, Soares AMVM (1998) Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned? Biomarkers 3: 157-163.
8. Soler F, Oropesa AL, Perez-Lopez M, Hernandez D, Garcia JP, et al. (2007) Acetylcholinesterase activity in seabirds affected by the Prestige oil spill on the Galician coast (NW Spain). Science of The Total Environment 372: 532-538.
9. Cordi B, Fossi C, Depledge M (1997) Temporal biomarker responses in wild passerine birds exposed to pesticide spray drift. Environmental Toxicology and Chemistry 16: 2118-2124.
10. Fossi MC, Massi A, Leonzio C (1994) Blood esterase inhibition in birds as an index of organophosphorus contamination: field and laboratory studies. Ecotoxicology: 11-20.
11. McInnes PF, Andersen DE, Hoff DJ, Hooper MJ, Kinkel LL (1996) Monitoring exposure of nestling songbirds to agricultural application of an organophosphorus insecticide using cholinesterase activity. Environmental Toxicology and Chemistry 15: 544-552.

12. Parsons KC, Matz AC, Hooper MJ, Pokras MA (2000) Monitoring Wading Bird Exposure to Agricultural Chemicals Using Serum Cholinesterase Activity. Environmental Toxicology and Chemistry 19.
13. Garcia LM, Castro B, Ribeiro R, Guilhermino L (2000) Characterization of cholinesterase from guppy (Poecilia reticulata) muscle and its in vitro inhibition by environmental contaminants. Biomarkers 5: 274-284.
14. Radic Z, Taylor P (2006) Structure and Function of Cholinesterases. In: Gupta RC, editor. Toxicology of Organophosphate and Carbamate Pesticides. Waltham: Elsevier Academic Press. pp. 161-186.
15. Monteiro M, Quintaneiro C, Morgado F, Soares AMVM, Guilhermino L (2005) Characterization of the cholinesterases present in head tissues of the estuarine fish Pomatoschistus microps: Application to biomonitoring. Ecotoxicology and Environmental Safety 62: 341-347.
16. Amat JA, Green AJ (2010) Waterbirds as Bioindicators of Environmental Conditions. In: Hurford C, Schneider M, Cowx I, editors. Conservation Monitoring in Freshwater Habitats: A Practical Guide and Case Studies. New York: Springer. pp. 45-52.
17. Diamond AW, Devlin CM (2003) Seabirds as indicators of changes in marine ecosystems: Ecological monitoring on Machias Seal Island. Environmental Monitoring and Assessment 88: 153-175.
18. Roy C, Grolleau G, Chamoulaud S, Rivere JL (2005) Plasma B-esterase activities in European raptors (vol 41, pg 184, 2005). Journal of Wildlife Diseases 41: 471-471.
19. Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity. Biochemical Pharmacology 7: 88-&.
20. Guilhermino L, Celeste Lopes M, Carvalho AP, Soares AM (1996) Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile Daphnia magna. Chemosphere 32: 727-738.
21. Eto M (1974) Organophosphorus Pesticides. Ohio: CRC Press.22. Austin L, Berry WK (1953) Two selective inhibitors of cholinesterase. Biochemical Journal 53: ix.
23. Sanchez-Hernandez JC (2007) Ecotoxicological Perspectives of B-esterases in the Assessment of Pesticide Contamination. In: Plattenberg RH, editor. Environmental Pollution: New Research. New York: Nova Science Publishers Inc. pp. 1-45.
24. Fildes K, Szabo JK, Hooper MJ, Buttemer WA, Astheimer LB (2009) Plasma cholinesterase characteristics in native Australian birds: significance for monitoring avian species for pesticide exposure. Emu 109: 41-47.

25. Moralev SN, Rozengart EV (2007) Specificity of Cholinesterases in Reaction with Substrates. In: Moralev SN, Rozengart EV, editors. Comparative enzymology of cholinesterases. San Diego, USA: International University Line. pp. 15-98.
26. Sanchez-Hernandez JC, Sanchez BM (2002) Lizard cholinesterases as biomarkers of pesticide exposure: Enzymological characterization. Environmental Toxicology and Chemistry 21: 2319-2325.
27. Goldstein MI, Lacher TE, Zaccagnini ME, Parker ML, Hooper MJ (1999) Monitoring and Assessment of Swainson's Hawks in Argentina Following Restrictions on Monocrotophos Use, 1996–97. Ecotoxicology 8: 215-224.

Chapter 3

Brain Cholinesterase Reactivation as a Marker of Pesticide Exposure in the Yellow-legged Gull Larus michahellis (Naumann, 1840): A Case Study.

3. Brain Cholinesterase Reactivation as a Marker of Pesticide Exposure in the Yellow-legged Gull *Larus michahellis* (Naumann, 1840): A Case Study.

3.1 Abstract

From small sparrows to medium-large seabirds, bird poisoning by organophosphate (OP) and carbamate (CB) pesticides has been extensively reported in literature. Amongst other, OP and CB poisoning has been observed to trigger a wide range of effects in avian species such as ataxia and ultimately death. From late 2010 to early 2011 an increased mortality in seagulls was observed along the northern coast of Portugal, with individuals' exhibiting neurologic disorders consistent with a scenario of pesticide poisoning. In order to clarify whether or not this increased mortality was related to OP and CB poisoning, cholinesterase (ChE) reactivation was tested in brain tissue of the yellow-legged gull (*Larus michahellis*) applying both chemical and spontaneous procedures.

Average initial brain cholinesterase activity in *L. michahellis* was 40.92±5.23 U/mg of protein. Following chemical and spontaneous reactivation assays, this activity was found to increase markedly, with a maximum average of 124.61±69.80 % (0.01 mM of 2-PAM) and 198.79±107.18 % (factor dilution of 10), respectively. ChE reactivation was found to decrease at increasing concentrations of 2-PAM and dilution factor. These results suggest that birds used for these analyses were exposed to both OP and CB pesticide compounds, inhibiting ChE activity up to 50% in some of the cases and impairing neurologic physiology, which explains the symptomatology observed in individuals. Additionally, the dissimilar performance of the reactivation assays amongst individuals' suggests a differential contribution of OP and CB to the acute effects observed on birds. In other words, CB exposure seems to have been the main cause of birds poisoning as in seven out of the eleven individuals analyzed, the percentage of spontaneous reactivation was markedly higher.

Keywords: Cholinesterase, spontaneous reactivation, 2-PAM, seagull.

3.2 Introduction

Present in the nervous system of both vertebrates and invertebrates, cholinesterases are a family of enzymes that catalyze the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid [1,2]. Nerve impulses pass across synapses through release of acetylcholine which, after the stimulating signals are transmitted, is readily hydrolyzed to allow other signals to pass. Acetylcholinesterase (AChE; EC 3.1.1.7), which serves to terminate synaptic transmission, belongs to this family of enzymes and is mostly found in the central nervous system and neuromuscular junctions [3]. In several avian species AChE has also been reported to be the predominant form present on brain [4].

Cholinesterase (ChE) activity is, though, particularly prone to inhibition by carbamate (CB) and organophosphate (OP) pesticides, which causes an over accumulation of acetylcholine in the nerve synapses, leading to nerve overstimulation which may trigger a wide range of adverse effects, from convulsions to paralysis, and ultimately death [1,5].

Amongst other *taxa*, birds are particularly vulnerable to OP and CB exposure, which may occur through ingestion of contaminated water and food, dermal contact and inhalation [6,7]. Avian poisoning by OP and CB has been extensively documented in the literature, from small Passeriformes such as the house sparrow (*Passer domesticus*) or the goldfinches (*Carduelis carduelis*) to medium-large water bird species such as seagulls (*Larus* spp.) and shags (*Phalacrocorax aristotelis*) [8].

Over the years, several studies have successfully used the inhibition of cholinesterase activity as a tool to diagnose OP and CB exposure, nonetheless this approach constrains some limitations as results obtained are often difficult to correlate with adverse effects at the organism level and/or when it is not possible to obtain reference activity values against which cholinesterase inhibition can be inferred [9]. Additionally, ChE activity may also be inhibited by other environmental contaminants such as petroleum-derived products, metals and detergents [1,10,11], which may act as confounding factors when diagnosing pesticide poisoning. As an alternative to this, the use of cholinesterase reactivation techniques has been suggested in literature to demonstrate acute ChE inhibition [9,12].

The inhibition of brain and plasma cholinesterases by OP and CB pesticides originates phosphorylated and carbamylated enzyme intermediates that might be reversed by using simple *in vitro* procedures [12,13]. Phosphorylated ChEs may have its activity restored chemically by nucleophilic reagents such as the oxime pyridine-2-aldoxime methiodide (2-PAM) [13-15]. Briefly, the partially electropositive nitrogen of 2-PAM is attracted to the anionic site of the ChE, which enables 2-PAM to attach to the site where the OP inhibitor

has previously attached and then attaches to the electronegative phosphorus atom, removing it from the enzyme and allowing its reactivation (Fig. 3.1). The specificity of 2-PAM nucleophilic properties toward OP compounds has been commonly accepted [12,16,17]. Nonetheless, the effectiveness of chemical reactivation by 2-PAM and other nucleophilic agents is reduced by the dealkylation (aging) of the phosphorilated enzyme [13].

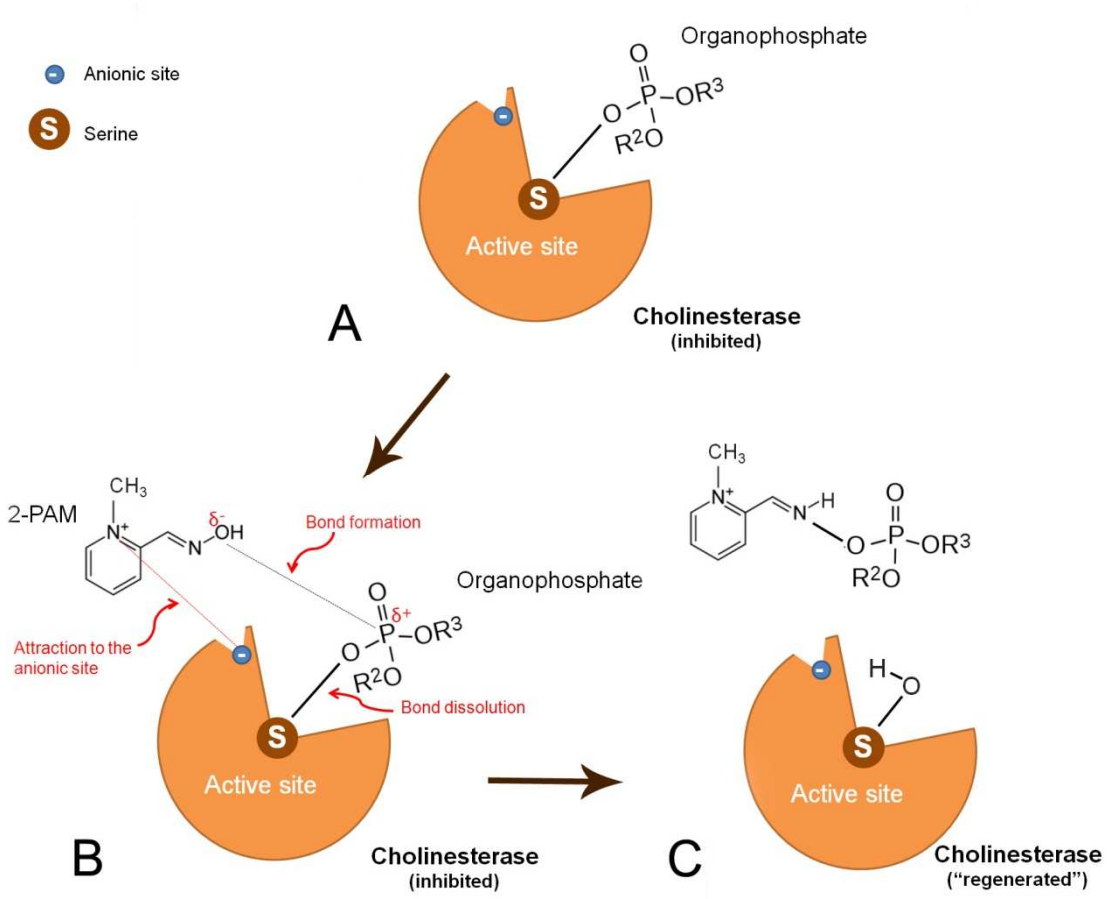

Fig. 3.1. Chemical reactivation of a phosphorilated ChE with 2-PAM. (A) OP inhibited enzyme, (B)-2-PAM attaches itself at the site the OP and its negatively charged atom of oxygen binds to the positively charged phosphorus atom of the OP, (C) The bond between the phosphorus atom and the oxygen of the serine is broken, and the OP molecule is removed from the ChE, leaving the "regenerated" enzyme ready to work normally again.

Carbamylated ChEs are generally less stable and therefore more prone to spontaneous hydrolysis. Reactivation of a carbamylated enzyme can be enhanced by sample dilution to minimize the effects of excess inhibitor, or by removing inhibitor through dialysis or gel filtration [5,12]. Contrarily to OP-inhibited ChEs, CB-inhibited ChEs do not reactivate with 2-PAM [12,14]. These differences have been used as the basis to distinguish between OP and CB exposure [14].

The main objective of this study was to investigate if the increased mortality of seagulls observed between late 2010 and early 2011 along the northern coast of Portugal was related to OP and CB poisoning. Symptomatology of individuals included, amongst others, diarrhoea and neurologic dysfunctions consistent with pesticide poisoning. In order to clarify this hypothesis it was the cholinesterase (ChE) reactivation was tested in brain tissue of yellow-legged seagull (*Larus michahellis*) individuals recovered from that mortality event, applying both chemical and spontaneous procedures

3.3 Materials and methods

3.3.1 Samples collection and preparation

All the animals submitted for analysis died before or during rehabilitation procedures in the veterinary centre of Gaia's Biological Park, a nature reserve located in Avintes (Porto, Portugal). After death, animals were frozen immediately at -20ºC and then transferred to the lab where they were kept at -80ºC until tissue dissection and analysis.

Brain samples used to measure ChE activity and reactivation were homogenised in phosphate buffer (0.1M, pH 7.2) using a Yastral GmbH d-7801 Dottingen homogenizer and kept on ice during the process. All enzymatic and reactivation assays were performed on the supernatant (S6) obtained by centrifugation (3 minutes, 6000 rpm) of homogenates at 4ºC.

3.3.2 Chemicals

Acetylthiocholine iodide, 5,5'-Dithiobis(2-nitrobenzoic acid), bovine γ-globulin and pyridine-2-aldoxime methochloride (2-PAM) were obtained from Sigma-Aldrich Europe (Netherlands). All the other chemicals used in this experiment were purchased from Merck (Germany).

3.3.3 Protein Quantification

The concentration of protein in all samples was determinated in quadruplicate by the Bradford method [18], adapted to microplate using bovine γ-globulin at 595 nm. Measurements were made using a Thermo Scientific Multiskan® Microplate reader from Spectrum.

3.3.4 ChE determinations

Cholinesterase (ChE) activity was determined in quadruplicate according to the Ellman method [19] adapted to microplate [20] using a microplate reader (Thermo Scientific Multiskan® Spectrum). Briefly, for each 50µl of sample (initial and after reactivation assays), 250µl of reaction mixture was added and after 10 minutes absorbance was measured three times, with a time lag of 5 minutes each, at 414 nm. Reaction mixture was prepared by mixing 30 ml of phosphate buffer (0.1M, pH 7.2) with 0.2 ml of acetylthiocholine iodide (0.075M) and 1ml of DTNB (10mM). For all analysis, a blank analysis made of ultrapure water was also assayed to account for reagents absorbance. The enzymatic activity was expressed in units (U) per mg of protein (1U is a µmol of substrate hydrolyzed per minute).

3.3.5 Reactivation assays

Reactivation of ChE was tested following an adaptation of the methods described by Martin *et al.* [14]. For the chemical reactivation assay, for each S6 brain homogenate one aliquot of 250-µl and seven aliquots of 200-µl were separated. The 250-µl aliquot was maintained on ice until assayed for initial ChE activity and the others were used for the reactivation assay. Six aliquots were incubated with 200µl of 2-PAM (concentrations ranging from 0.01-5 mM) and one with 200µl of ultrapure water (water control), for one hour at 25ºC. ChE activity was then measured as described above. To account for the differences of absorbance produced by 2-PAM during the enzymatic assay, an additional blank composed of 200µl of ultrapure water and 200µl 2-PAM was prepared for each concentration of 2-PAM used and incubated in the conditions above mentioned.

In order to study spontaneous reactivation, each S6 brain homogenate was separated into two additional 250-µl sub-samples. The first aliquot was kept on ice and the second aliquots were successively diluted 10, 20, 40, 80 and 100 times with cold phosphate buffer (0.1M, pH 7.2) up to a final volume of 500 µl. Diluted samples and the undiluted aliquot were then incubated at 4ºC for 24h and ChE activity was determined.

3.3.6 Set up, data treatment and analysis

Enzymatic activity was determined for each sample before (i.e initial activity) and after the reactivation assays. In samples assayed for chemical reactivation, absorbance of samples during the enzymatic reaction was corrected by subtracting the absorbance

obtained in the additional blanks prepared with 2-PAM. The absorbance of all other samples was corrected using the blank of ultrapure water.

Percentage of reactivation was calculated by converting the activity obtained in the reactivation assays into percentages, using the following formula:

$$\% \text{ of Reactivation} = [(ChE_R \times 100) / ChE_I] - 100$$

ChE_R = ChE activity after reactivation
ChE_I = Initial ChE activity

Normality of variables was checked using the Shapiro-Wilk Test. Data were found to deviate significantly from normality and since normalizing was not possible for all data, all statistical analysis performed were non-parametric. A Kruskal-Wallis analysis of variance (ANOVA on Ranks) was performed to compare differences between 2-PAM concentrations (chemical reactivation) and dilution factors (spontaneous reactivation). All data analyses were performed using SigmaPlot® 11 software (Systat Software Inc.).

3.4 Results

In order to investigate ChE reactivation in brain of *L. michahellis*, we assayed ChE activity before and after chemical and spontaneous reactivation using increasing concentrations of the oxime 2-PAM (chemical reactivation) or by successive dilutions of brain samples (spontaneous reactivation) (Fig. 3.1). Maximum enzyme reactivation in the chemical and spontaneous assays was obtained at 0.01 mM of 2-PAM (124.61±69.80 %) and after a factor dilution of 10 (198.79±107.18 %), respectively.

Percentage of ChE reactivation was observed to decrease significantly ($P>0.05$) with increasing concentrations of 2-PAM. Percentage of ChE reactivation was also observed to decrease, although at no significant extent, with increasing dilution factor (DF), except at the highest dilution factor (DF=100) in which percentage of reactivation increased. Minimum reactivation percentages were registered in the chemical assay at 5 mM of 2-PAM (16.471±37.95 %) and in the spontaneous assay after applying a dilution factor of 80 (21.78±43.08 %).

Brain initial and reactivated ChE activities are depicted on Table 3.1. Average initial brain activity in *L. michahellis* was 40.92±5.23 U/mg of protein. Brain ChE did not vary greatly when incubating the samples for 24h without dilution (average activity, 39.46±3.48

U/mg of protein). After dilution, however, brain ChE was found to increase steadily (average activity, 99.25±39.71 U/mg of protein), having been observed in some cases increases of about 423.7%, 867.0% and 869.9% for birds recorded as no. 1, 7 and 9 respectively. Lowest percentages of reactivation following dilution were 0% (record birds' no. 6, 8 and 10) and 5.3% (record bird no. 11).

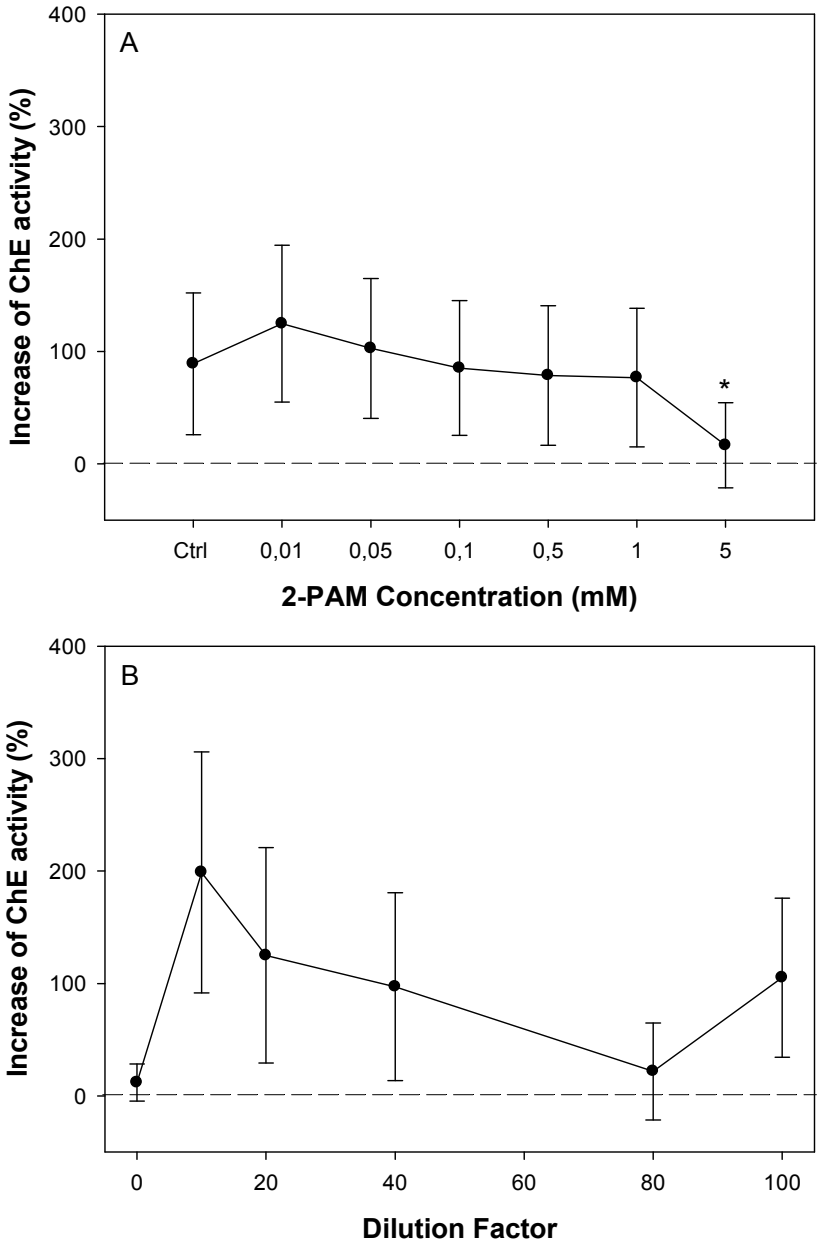

Fig. 3.2. Performance of chemical (A) and spontaneous (B) reactivation assays in brain ChE of *Larus michahellis*. Results are expressed as the mean ± SE of eleven birds, Ctrl= ultrapure water control, *= significantly different from control (Dunn's test, P<0.05).

Regarding the chemical reactivation assay, generally no effect on brain ChE activity was observed when comparing the ultrapure water control (average activity, 55.32±5.85 U/mg of protein) with the initial brain ChE activity (P>0.05). This was true for all individuals analysed, except for recorded birds no. 1, 7 and 9 in which an increase of ChE activity was observed after incubating the samples with the ultrapure water control for one hour at 25ºC. This increase was, most probably, result of spontaneous reactivation prompted by the dilution of samples with ultrapure water. This same effect could be the reason why the average percentage of reactivation of the 2-PAM control (89.03±63.04 %) was higher than the control of the spontaneous assay (11.84±16.50 %) (Fig. 3.2). After sample incubation with 0.01 mM 2-PAM, in most of the individuals analysed, brain ChE activity was also found to increase (not significantly) when comparing to initial ChE activity (63.74±9.83 U/mg of protein). Maximum percentage of reactivation following samples incubation was about 290%, 410% and 667%, for recorded birds no. 1, 10 and 7, respectively. Lowest reactivation percentages in the chemical assay were 0% (recorded birds' no. 3, 8 and 11) and 7.4% (recorded bird no. 6).

Table 3.1. Brain ChE activity of *Larus michahellis* before (initial activity) and after reactivation assays. Activities are expressed as U/mg of protein and values in brackets correspond to the percentage of reactivation. Averages of activities are presented by mean ± (SE). a= enzymatic activity obtained when incubating the samples with ultrapure water (1 hour, 25ºC), b=enzymatic activity obtained after incubating the original (not diluted) samples for 24h, 4ºC.

Bird Record (no.)	Initial activity	Chemical Reactivation		Spontaneous Reactivation	
		Water control [a]	0.01 mM 2-PAM (%)	Control after 24h [b]	1:10 (%)
1	24.48	82.51	95.50 (290)	34.69	128.18 (423.7)
2	44.48	48.40	52.85 (18.8)	46.74	56.63 (27.4)
3	52.11	50.69	46.15 (0)	46.53	63.31 (21.5)
4	44.29	52.54	57.81 (30.5)	43.55	62.03 (40.0)
5	37.51	41.06	41.18 (9.79)	42.34	54.59 (45.5)
6	42.21	43.36	45.33 (7.4)	34.50	39.99 (0.0)
7	10.61	82.97	81.35 (667)	17.68	102.57 (867.0)
8	77.59	71.67	73.31 (0)	44.25	49.41 (0.0)
9	50.00	72.22	68.38 (36.8)	32.31	484.61 (869.9)
10	25.92	22.78	132.17 (410)	63.13	7.36 (0.0)
11	40.91	40.31	7.11 (0)	28.38	43.08 (5.3)
Average activity	40.92±5.23	55.32±5.85	63.74±9.83	39.46±3.48	99.25±39.71

3.5 Discussion

In order to investigate if an increased mortality of seagulls observed along the northern coast of Portugal between 2010 and 2011 was related to OP and CB poisoning, in this study both chemical and spontaneous ChE reactivation techniques were tested in the brain of deceased *L. michahellis* individuals recovered from that mortality event. The use of ChE activity as a biomarker to diagnose exposure of organisms to anticholinesterase compounds such as OP and CB pesticides has been routinely described in previous works [17,21,22]. This approach usually requires reference or basal activity values from healthy animals to infer cholinesterase inhibition which in some cases is not available, reason why ChE reactivation techniques haven been proposed as an alternative to identify OP and CB exposure [9,12].

The methodologies used in this work were based on the original work described by Martin *et al.* [14]. Enzyme kinetics are known to vary significantly amongst species and tissues, therefore in order to determine best assay conditions for chemical and spontaneous assays brain ChE reactivation was tested in *L. michahellis* under different concentrations of 2-PAM and sample dilutions, respectively. In this study, the enzymatic activity measured in brain showed an increase with lower concentrations of 2-PAM, but with no statistical significance, possibly as a consequence of the high heterogeneity of enzymatic activities reported, which could be due to dissimilar background field conditions. Namely, birds studied might have been exposed to other sources of contamination (e.g. metal contamination) which might have induced additional stress on the exposed individuals, therefore triggering dissimilar physiological responses and acting as a confounding factor.

This absence of significance was true for all 2-PAM concentrations, except for 5 mM where the percentage of enzyme reactivation was reported to be significantly lower than the control, which is indicative of enzyme inhibition. Such inhibitory effect of 2-PAM has already been reported for fish and crabs' AChE activities at 0.1 mM, and for plasma butyrylcholinesterase (BChE) of starlings exposed to demetonS-methyl after incubation with 12.5 mM of 2-PAM [23,24]. On the other hand, a 2-PAM concentration of 0.2mM was found to be the optimum concentration for reactivating plasma BChE activity on OP-exposed lizards [25]. Testing chemical reactivation with 2-PAM at different concentrations is, thus, of most importance, as it allows to optimize the assay conditions and increase the efficiency of the chemical reactivation. Even though in this particular case no consistent statistical data was obtained, higher enzymatic recovery was observed when using

0.01mM of 2-PAM; thus in further studies with brain ChE of *L. michahellis* it seems legitimate to conclude that this concentration seems to be the most appropriate to retrieve a better chemical reactivation performance.

Optimization of spontaneous ChE reactivation was achieved by applying different dilution factors (1:10 to 1:100) to the brain samples analysed. Best performance of spontaneous reactivation was achieved at lower dilution factors (DFs) and, similarly to the chemical assay, no statistical differences between the different DFs tested and the assay control (DF=0) were reported. As above mentioned, this might have been a result of the elevated heterogeneity observed in the enzymatic activities as a result of the dissimilar environmental conditions that birds may have been exposed in the field. Despite the absence of statistical significancy to infer optimal DF to use in future studies, higher percentage of ChE reactivation was observed when diluting the brain samples in a DF of 1:10; therefore, in order to retrieve a better performance of the spontaneous reactivation in future studies with brain ChE of *L. michahellis*, it is further recommended the use of this DF. Considering these previously discussed assay conditions as optimal, herein all values presented will be relative to the results obtained under these same conditions.

Generally, average initial brain activity in *L. michahellis* (40.92±5.23 U/mg of protein) was found to increase following chemical (55.3±25.85 U/mg of protein) and spontaneous reactivation (99.26±39.71 U/mg of protein) assay, with the percentage of enzyme activity being restored up to 30 and 50%, respectively (Table 3.1). In literature it has been usually accepted that brain ChE activity depressions of about 20% in birds, fish and invertebrates were indicative of exposure to anti-ChE compounds, while depressions greater than 50% in dead animals has been considered as diagnostic of death due to ChE inhibition [9,16]. Complete reactivation of all inhibited enzyme is often difficult to conclude. Amongst other factors that could act as confounding factors (e.g. aging of phosphorilated enzyme, presence of other type of ChE-inhibiting compound), as concluded by the optimization assay incubation conditions influence performance of ChE reactivation, thus it is likely that even optimized conditions may not lead to complete reactivation of all inhibited enzyme. Fildes et al. [21] have suggested a formula[1] to account for percentage of inhibition, which considering the aspects above mentioned, would represent a minimum level of inhibition. Applying that same formula[1] for each individual analysed (percentage of reactivation used to make the conversion was always the highest value of both assays), percentage of inhibition was observed to vary between 17.7 to 89.7%. It seems, therefore, reasonable to suggest that the birds analyzed were exposed to both OP and CB pesticides, up to

[1] $\%\ inhibition = \left(\frac{\%\ reactivation}{100+\%reactivation}\right) \text{x}\ 100$

concentrations high enough to induce impaired neurologic physiology, thus explaining the symptoms observed in these individuals. These observations were true for the majority of the birds studied except for gulls no. 6, 8 and 11 which showed both spontaneous and chemical brain ChE reactivations never higher than 10%. This lack of reactivation could be related to the aging of OP-inhibited ChEs, as this process is known to inhibit irreversibly enzymes, even in the presence of 2-PAM [13,14] or to the previous exposure of individuals to other classes of ChE-Inhibiting compounds (e.g. metals, detergents) [1,10].

Moreover, analysis of reactivation data for both chemical and spontaneous assays depicted on Table 3.1 allowed us to discriminate individual differences in the performance of both assays amongst animals analyzed. For example, while on gulls no. 1, 7 and 9 brain ChE activity was mainly reactivated spontaneously, with percentage of reactivation varying from 423.7 to 869.9%, brain ChE of gull no. 10 was found to reactivate more readily after incubation with 2-PAM (percentage of reactivation=410%). This might be indicative of a differential contribution of OP and CB exposure to the acute effects observed on the studied gulls, with CB exposure being most likely the main cause of poisoning as, in seven out of the eleven individuals analyzed, the percentage of reactivation obtained spontaneously was markedly higher than with 2-PAM.

In conclusion, the variability of brain ChE reactivation under different assay conditions underscores the importance of optimizing chemical and spontaneous assay conditions prior to its use as a marker of OP and CB exposure on birds. In this study we were able to successfully reactivate brain ChE of *L. michahellis* up to 20 and 50%, which has been accepted in the literature as indicative of exposure to ChE-inhibiting compounds and acute toxicity, respectively. Additionally, using both spontaneous and chemical assays allowed us to distinguish between OP and CB inhibition, enabling us to infer that CB exposure may have been the principal cause of death of birds analyzed. In order to further use the reactivation assays in brain ChE of *L. michahellis*, it is recommended the use of 2-PAM at 0.01mM (chemical reactivation) and a DF of 10 (spontaneous reactivation) as maximum percentages of ChE reactivation were reported under these assay conditions.

3.6. Acknowledgements

This work has been carried out thanks to the contribution of Gaia Biological Park (Avintes, Portugal), in particular the Centre for Rehabilitation of Wild Animals, who kindly provided us all the brain samples. The Portuguese Foundation for Science and Technology supported the postdoctoral fellowship of M. S. Monteiro (FCT/SFRH/BPD/45911/2008).

3.7. References

1. Frasco MF, Fournier D, Carvalho F, Guilhermino L (2005) Do metals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. Biomarkers 10: 360-375.

2. Voet D, Voet JG (1990) Biochemistry. New York: Jonh Wiley. XVII, 1223 p.

3. Radic Z, Taylor P (2006) Structure and Function of Cholinesterases. In: Gupta RC, editor. Toxicology of Organophosphate and Carbamate Pesticides. Waltham: Elsevier Academic Press. pp. 161-186.

4. Osten JRV, Soares A, Guilhermino L (2005) Black-bellied whistling duck (Dendrocygna autumnalis) brain cholinesterase characterization and diagnosis of anticholinesterase pesticide exposure in wild populations from Mexico. Environmental Toxicology and Chemistry 24: 313-317.

5. Manahan SE (2003) Toxicological Chemistry and Biochemistry. Boca Raton (FL): Lewis Publishers.

6. Hill EF (2003) Wildlife Toxicology of Organophosphorus and Carbamate Pesticides. In: Hoffman DJ, Rattner BA, Burton GA, Cairns J, editors. Handbook of Ecotoxicology. Second ed. Boca Raton: Lewis Publishers. pp. 281-312.

7. Fleischli MA, Franson JC, Thomas NJ, Finley DL, Riley W (2004) Avian mortality events in the united states caused by anticholinesterase pesticides: A retrospective summary of National Wildlife Health Center records from 1980 to 2000. Archives of Environmental Contamination and Toxicology 46: 542-550.

8. Guitart R, Sachana M, Caloni F, Croubels S, Vandenbroucke V, et al. (2010) Animal poisoning in Europe. Part 3: Wildlife. Veterinary Journal 183: 260-265.

9. Escartín E, Porte C (1996) Acetylcholinesterase Inhibition in the Crayfish Procambarus clarkii Exposed to Fenitrothion. Ecotoxicology and Environmental Safety 34: 160-164.

10. Guilhermino L, Barros P, Silva MC, Soares AMVM (1998) Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned? Biomarkers 3: 157-163.

11. Soler F, Oropesa AL, Perez-Lopez M, Hernandez D, Garcia JP, et al. (2007) Acetylcholinesterase activity in seabirds affected by the Prestige oil spill on the Galician coast (NW Spain). Science of The Total Environment 372: 532-538.

12. Stansley W (1993) Field Results Using Cholinesterase Reactivation Techniques to Diagnose Acute Anticholinesterase Poisoning in Birds and Fish. Archives of Environmental Contamination and Toxicology 25: 315-321.

13. Trudeau S, Cartier GS (2000) Biochemical Methods to Determine Cholinesterase Activity in Wildlife Exposed to Pesticides. Québec, Canada: Canadian Wildlife Service.

14. Martin AD, Norman G, Stanley PI, Westlake GE (1981) Use of Reactivation Techniques for the Differential-Diagnosis of Organo-Phosphorus and Carbamate Pesticide Poisoning in Birds. Bulletin of Environmental Contamination and Toxicology 26: 775-780.

15. Thompson HM (1999) Esterases as markers of exposure to organophosphates and carbamates. Ecotoxicology 8: 369-384.

16. Goldstein MI, Lacher TE, Woodbridge B, Bechard MJ, Canavelli SB, et al. (1999) Monocrotophos-induced mass mortality of Swainson's hawks in Argentina, 1995-96. Ecotoxicology 8: 201-214.

17. Hooper MJ, Detrich PJ, Weisskopf CP, Wilson BW (1989) Organophosphorus Insecticide Exposure in Hawks Inhabiting Orchards during Winter Dormant-spraying. Bulletin of Environmental Contamination and Toxicology 46: 651-659.

18. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochemistry 72: 248-254.

19. Ellman GL, Courtney KD, Andres V, Jr., Feather-Stone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7: 88-95.

20. Guilhermino L, Celeste Lopes M, Carvalho AP, Soares AM (1996) Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile Daphnia magna. Chemosphere 32: 727-738.

21. Fildes K, Astheimer LB, Story P, Buttemer WA, Hooper MJ (2006) Cholinesterase response in native birds exposed to fenitrothion during locust control operations in eastern Australia. Environmental Toxicology and Chemistry 25: 2964-2970.

22. Hunt KA, Hooper MJ, Littrell EE (1995) Carbofuran Poisoning in Herons - Diagnosis Using Cholinesterase Reactivation Techniques. Journal of Wildlife Diseases 31: 186-192.

23. Monserrat JM, Bianchini A (2000) Methodological and biological aspects to be considered in acetylcholinesterase reactivation assays using 2-PAM. Environmental Toxicology and Pharmacology 9: 39-47.

24. Thompson HM, Walker CH, Hardy AR (1991) Inhibition of avian esterases by organophosphorus insecticides: Problems of reactivation and storage. Archives of Environmental Contamination and Toxicology 20: 509-513.

25. Sanchez-Hernandez JC, Sanchez BM (2002) Lizard cholinesterases as biomarkers of pesticide exposure: Enzymological characterization. Environmental Toxicology and Chemistry 21: 2319-2325.

Chapter 4

Ciconiiformes as Sentinels of Chemicals' Genotoxic Effects: A 5-Year Study on Portuguese Birds.

4. Ciconiiformes as Sentinels of Chemicals' Genotoxic Effects: A 5-Year Study on Portuguese Birds

4.1. Abstract

Over the past decades, the presence of micronucleated blood cells has been used as a widespread tool to detect genotoxic effects of xenobiotics in mammals, fish, amphibians and birds. In order to: (i) investigate the robustness of the Erythrocyte nuclear abnormalities (ENA) assay as a complementary analysis of the micronucleus test in Ciconiiformes, (ii) monitor the variation of occurrence of genotoxic effects in Portuguese populations of Ciconiiformes, and (iii) assess genotoxic recovery following bird rehabilitation, the frequency of micronucleus (MN) and other nuclear abnormalities (lobed, segmented, kidney-shaped and notched nuclei) was assessed in wild individuals of *Ciconia ciconia*, *Ardea cinerea, Ardea purpurea* and *Egretta garzetta*. About 80 blood smears, collected between 2007 and 2011 from birds from different geographic origins, were analysed and statistical tests were performed to determine potential temporal and geographical differences in the frequency of MN and other nuclear abnormalities.

Results showed high levels of MN and other nuclear abnormalities, and the sum of frequencies of all other abnormalities scored was generally higher than MN on the samples studied. Significant differences in the frequency of total nuclear abnormalities (TNA) were observed also between different years and geographical origins. These results suggest that the assessment of TNA, rather than only MN frequencies, may be a useful complementary tool for the study of induced genotoxicity in birds by chemical exposure.

Keywords: Genotoxicity, birds, micronucleus test, ENA assay

4.2. Introduction

Within a cell, the DNA molecule undergoes cyclic changes on its structure that makes it flux between a functionally stable double-stranded entity and an intermediate state, in which the double helix is partially uncoiled to allow DNA replication. During the latter state, the DNA molecule is less stable and thereby particularly susceptible to genotoxic damage [1].

Genotoxic damage refers to damage induced in the genetic material of cells when exposed to genotoxicant compounds. This damage, triggered by the disruption of the DNA replicating processes with consequent loss of its structural integrity, may include a wide range of effects from chromosomal aberrations to the initiation of carcinogenicity in individuals or hereditary defects that pass via germ cell mutations and teratogenicity [1,2]. Ultimately, these may alter individual fitness, reproductive success and, consequently, population dynamics [2].

Because genotoxic compounds may have such deleterious effects upon population dynamics, thereby making it an issue of major concern for conservation biology, assessing genotoxicity in the wild is of major importance and requires the choice of suitable and sensitive markers [2,3]. Over the years, a wide number of techniques derived from human cancer risk assessment has been employed to monitor genotoxic effects on the environment in a broad range of organisms, both *in vivo* and *in vitro*, including markers of DNA modifications (e.g. DNA adducts), markers of cytogenetic effects (e.g. micronuclei and chromosomal aberrations) and mutations [4,5].

Two examples of cytogenetic assays that can be used to detect genotoxic effects upon organisms are the Micronucleus test and the Erythrocyte Nuclear Abnormalities (ENA) assay. The Micronucleus test is based on the detection of micronuclei (MN), which are small cytoplasmic bodies that are produced as a consequence of genetic damage or impaired function of the spindle apparatus, and that has been used as a tool to detect genotoxic effects induced by environmental contaminants in mammals, fish, amphibians and birds [6]. Similarly, the ENA assay follows the same principle as the micronucleus test but instead of only using the frequency of micronuclei, this assay also includes the analysis of the frequency of other abnormal nuclear structures in mature erythrocytes, such as lobed or segmented nuclei. The ENA assay has already been widely applied to fish [7].

Although the selection of appropriate techniques is important to monitor genotoxicity on the environment, an aspect as equally important as the assay chosen, is the selection of

representative organisms as sentinels [2,3].Due their trophic level position, avian species such as those of the order Ciconiiformes (e.g. storks and herons) are particularly good indicators of environmental health. As long-lived species, located at upper trophic levels of both aquatic and terrestrial ecosystems, Ciconiiformes are good indicators as they are particularly prone to bioaccumulate organic contaminants present in the ecosystems, including genotoxins, which may cause stress and impair their fitness and survival [1,3,6]. Moreover, Ciconiiformes are very conspicuous and abundant species, with a basic ecology and habitats preferences well established, being therefore a relatively easy and inexpensive species to monitor [8].

With the main aims of (i) investigating the robustness of the Erythrocyte nuclear abnormalities (ENA) assay as a complementary analysis of the micronucleus test in Ciconiiformes, (ii) monitor the variation of genotoxic effects in Portuguese populations of Ciconiiformes, and (iii) assess genotoxic recovery following bird rehabilitation, in the present study the frequency of micronucleus (MN) and other nuclear aberrations in peripheral blood erythrocytes was assessed in Ciconiiformes. Four species were used: the Grey Heron (*Ardea cinerea*) and the Purple Heron (*Ardea purpurea*), two piscivorous birds, and the White Stork (*Ciconia ciconia*) and the Little Egret (*Egretta garzetta*), which have more generalist habits, feeding opportunistically on insects, amphibians, reptiles, earthworms and fish. Blood samples obtained from individuals with different life-history aspects (e.g. age, geographical origin, year), were taken from birds admitted at a wildlife rehabilitation centre, immediately upon arrival and whenever possible, during recovery until their release back into the wild.

This approach has important advantages. Firstly, using blood as a matrix to assess genotoxic damage allows to screen for recent exposure effects, as erythrocytes have a short lifespan (28-45 days) [9], and they do not undergo mitosis, which will reduce misinterpretation errors in the analysis of nuclei shape associated with nuclei division.. Secondly, by taking into account individuals' intrinsic aspects such as age, it is possible to infer whether or not these aspects could influence amount of genotoxic damage detected and act as confounding factors. Thirdly, by using (when possible) the pattern of recovery in the genotoxic damage, we could also assess basal levels of genotoxic damage, in a controlled environment. To our knowledge, the present study is the first to perform *in vivo* the ENA assay in mature peripheral erythrocytes, in order to study spatial and temporal variation of DNA damage in wild populations of Portuguese birds.

4.3. Material and methods

4.3.1. Birds sampled and blood collection

All birds used in this study were individuals admitted for rehabilitation at CERVAS (Centro de Ecologia, Recuperação e Vigilância de Animais Selvagens), a wildlife rehabilitation centre located in Gouveia (Guarda, Portugal). Blood samples, collected by the veterinary centre, were drawn from the brachial vein and used to prepare a blood smear. Samples of blood were only taken when birds didn't evidence any sign of pain or stress in order to prevent any harmful effects. Disturbance stress caused by the animal handling was minimized by limiting the handling period and using a small mantle to cover the head.

All procedures involving live bird handling were conducted according to the Guide for the Care and Use of Laboratory Animals of the European Union - in Portugal represented by Decreto de Lei nº 129/92 de 06 de Julho, Portaria nº 1005/92 de 23 de Outubro de 1992.

4.3.2. Slide preparation and genotoxic damage scoring

Genotoxic damage was assessed in mature peripheral erythrocytes using the ENA assay, as adapted by Pacheco and Santos [10]. Briefly, one blood smear per individual was fixed in methanol, stained with Diff-Quik and air dried. After this procedure, slides were then coded and scored blindly, and from each smear, 1000 erythrocytes were counted using 1000x of magnification in order to determine the frequency of the following nuclear abnormalities (Fig. 4.1): micronuclei (MN), lobed nuclei (L), kidney-shaped nuclei (K), segmented nuclei (S) and notched nuclei (N).

4.3.3. Statistical analysis

Normality of variables was checked using the Shapiro-Wilk Test. Data were found to deviate significantly from normality and since forcing a normalization of data would introduce additional error to posterior analysis, we preferred to use non-parametric statistics. A Spearman rank order correlation was performed to study the relationship between each category of abnormalities scored and the total frequency of abnormalities (TNA) observed.

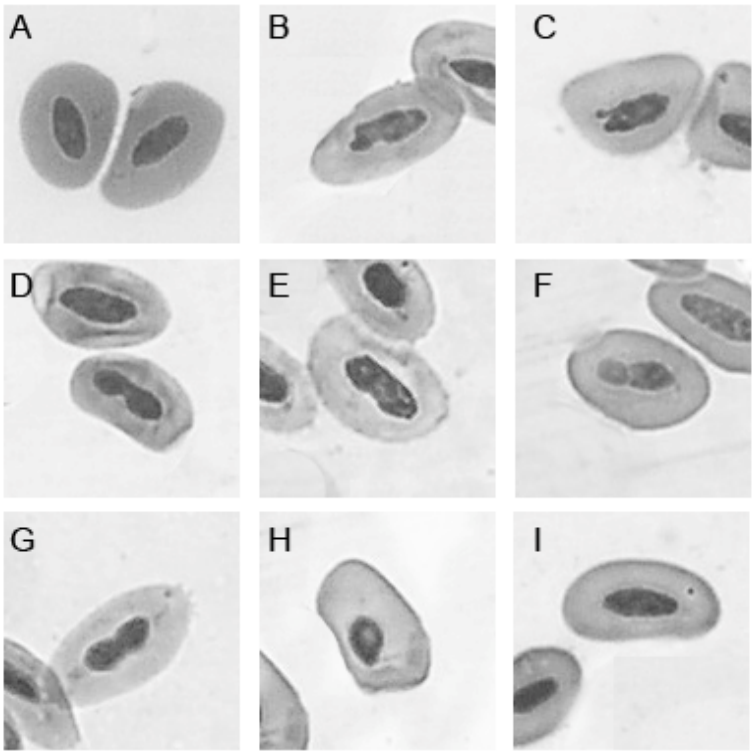

Fig. 4.1. Erythrocyte nuclear abnormalities (NA) in *Ciconia ciconia*: A) normal nuclei; B-C) lobed; D-E) Kidney shaped; F-G) Segmented; H) notched; I) Micronuclei.

Because of the large size and heterogeneity of the dataset, an exploratory analysis was performed using a Principal Component Analysis (PCA). The PCA was carried out in order to explore and highlight the relationships and patterns between samples, as well as to investigate which were the most important variables (among 'age', 'district', 'year', 'survival') in explaining the separation obtained among the different samples. Categorical (qualitative) variables (e.g. survival or geographical origin) were converted to a numerical scale, acknowledging however a certain degree of error associated to such a transformation. When possible, a Kruskal-Wallis analysis of variance (ANOVA on Ranks) was performed to compare differences between geographical and temporal origin of birds, as well as to discriminate age differences. A Mann-Whitney rank sum test was used to compare differences in the extent of DNA damage and the survival of individuals. Multivariate and correlation analysis were performed using XLSAT® statistical analysis add-in (Addinsoft™). All the other analyses were conducted using SigmaPlot® 11 software (Systat Software Inc.).

4.4. Results

4.4.1 Micronucleus (MN) and total nuclear abnormalities (TNA) frequencies in Ciconiiformes

Using the ENA assay to assess genotoxic effects on wild populations of four Ciconiiformes' species, micronuclei (MN) and other nuclear abnormalities were observed in all tested species. MN frequency in individuals tested was generally within the interval reviewed in the literature as normal for healthy Ciconiiformes and other avian orders [6,11,12], except in 23 cases in which MN frequencies were reported to be above the normal level of 2.14 MN/1000 erythrocytes in *C. ciconia* (22 cases) and *E. garzetta* (1 case) (Fig. 4.2). TNA was significantly higher on *C. ciconia* and *E. garzetta* than in *A. purpurea* and *A. cinerea* (Mann-Whitney Rank sum test, $P<0.05$). Lobed nuclei were the type of lesion most commonly reported for all species accounting for 74.5 to 86.1% of the anomalies scored. At a lower degree, segmented nuclei was generally the second most scored lesion accounting for 3.7 to 13.8% of the anomalies, followed by micronuclei (3.4 to 9.9%), kidney-shaped nuclei (0 to 4.4%) and notched nuclei (0 to 0.4%). MN and TNA frequencies are depicted on Table 4.1.

Analysis of correlation was performed by comparing individual frequency of scored abnormalities with TNA frequency and is presented on Table 4.2. Coefficients of correlation greater than 0.8 would describe strong correlations while values inferior to 0.5 would describe the weak ones. Accordingly, a strong and significant ($P<0.05$) correlation between lobed nuclei (L) and total nuclear abnormalities (TNA) was reported for *C. ciconia*, *A. cinerea* and *A. purpurea* (coefficients of correlation: 0.954, 0.968 and 0.955, respectively). A significant ($P<0.05$), but not strong correlation between TNA and segment (S), kidney-shaped (K) and micronuclei (MN) was also observed in *C. ciconia* (coefficients of correlation: 0.626, 0.410 and 0.417, respectively), nonetheless no correlation of TNA frequency against the other categories of anomalies scored was identified in *A. cinerea* and *A. purpurea*. No correlation analysis was possible for *E. garzetta*, as the sample size of slides analysed was too low (n=2). Sample size of analysis in *A. cinerea* and *A. purpurea* was increased by using blood samples taken during rehabilitation of individuals, as temporal variation of nuclear abnormalities was unlikely to modify relative proportion of the categories of abnormalities identified and TNA frequency.

Table 4.1. Total nuclear abnormalities (TNA) and micronuclei (MN) frequency per 1000 erythrocytes in Ciconiiformes. When n>2, values are presented by the mean and standard error is given in brackets; *x* and *y* are used to present depicted values of TNA and MN frequencies when n=2

		Ciconia ciconia			***Ardea cinerea***		
Year	**District**	**n**	**TNA**	**MN**	**n**	**TNA**	**MN**
2007	**Guarda**	1	5.0	2.0	2	*x*= 25.0 *y*= 17.0	*x*= 6.0 *y*= 2.0
	Portalegre	2	*x*= 2.0 *y*= 8.0	*x*= 0.0 *y*= 0.0	-----	-----	-----
2008	**Bragança**	-----	-----	-----	-----	-----	-----
	Coimbra	4	7.2 (5.0)	0.8 (0.8)	-----	-----	-----
	Guarda	1	9.0	0.0	1	17.0	2.0
	Portalegre	1	14.0	0.0	1	54.0	0.0
	Viseu	-----	-----	-----	2	*x*= 12.0 *y*= 7.0	*x*= 2.0 *y*= 1.0
2009	**Aveiro**	2	*x*= 10.0 *y*= 17.0	*x*= 0.0 *y*= 0.0	-----	-----	-----
	Bragança	1	47.0	0.0	-----	-----	-----
	Coimbra	7	46.6 (8.6)	2.6 (1.5)	1	46.0	5.0
	Guarda	5	39.8 (4.2)	1.4 (0.7)	2	*x*= 140.0 *y*= 11.00	*x*= 0.0 *y*= 1.0
	Portalegre	6	47.7 (5.5)	8.7 (3.9)	-----	-----	-----
2010	**Aveiro**	-----	-----	-----	1	24.0	0.0
	Bragança	-----	-----	-----	-----	-----	-----
	Coimbra	6	83.3 (15.6)	5.3 (3.4)	-----	-----	-----
	Guarda	4	73.0 (20.2)	1.0 (0.7)	1	31.0	1.0
	Leiria	-----	-----	-----	1	5.0	1.0
	Portalegre	3	82.0 (8.5)	2.0 (0.6)	1	33.0	0.0
2011	**Coimbra**	3	84.3 (15.7)	17.7 (7.5)	-----	-----	-----
	Guarda	2	*x*= 39.0 *y*= 64.0	*x*= 0.0 *y*=36.0	-----	-----	-----
	Portalegre	4	29.8 (7.9)	2.5 (1.3)	-----	-----	-----

		Ardea purpurea			***Egretta garzetta***		
		n	**TNA**	**MN**	**n**	**TNA**	**MN**
2008	**Bragança**		-----	-----	1	62.0	3.0
2009	**Coimbra**	2	*x*= 41.0 *y*= 15.0	*x*= 0.0 *y*= 1.0	-----	-----	-----
	Guarda	-----	-----	-----	1	25.0	0.0

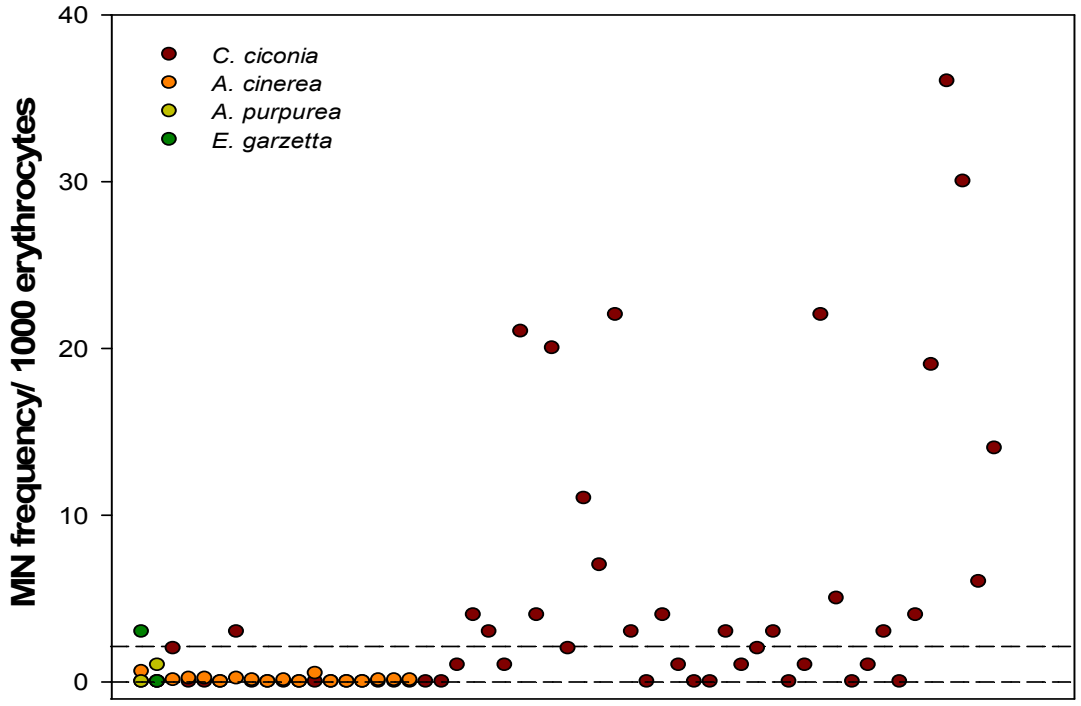

Fig. 4.2. Variability of micronuclei (MN) frequency in Portuguese populations' of Ciconiiformes and its deviation from the minimum (0) and maximum (2.14) normal values of MN (depicted by the dashed lines) reported in the literature for healthy individuals [6,11,12].

Table 4.2. Spearman correlation coefficients of different categories of nuclear abnormalities scored and the total nuclear abnormalities (TNA) for each studied species. Values highlighted correspond to statistical significant values (P<0.05). Sample size (n) of *Ciconia ciconia, Ardea cinerea* and *Ardea purpurea* was 57, 18 and 7, respectively; L= lobed, S= segmented, K= kidney-shaped, N= notched, MN= micronuclei.

Species	L	S	K	N	MN	
Ciconia ciconia	0.954	0.626	0.410	-0.008	0.417	**TNA**
Ardea cinerea	0.968	0.230	0.412	------	-0.160	**TNA**
Ardea purpurea	0.955	0.267	0.435	------	0.356	**TNA**

4.4.2. Variability of genotoxic effects in Portuguese populations of Ciconiiformes

Spatial and yearly variability in the frequency of MN and TNA was observed in *C. ciconia,* with PC1 and PC2 generally accounting for over 73% of the variability in the dataset (Fig. 4.3.). PCA showed a segregation of points into three main clusters (dashed blue lines). The year and district of collection of the birds showed a strong influence in the

frequency of MN and TNA ('Year' was positively correlated to PC1, explaining on its own >47% of the information in the data, while 'District' was positively correlated to PC2, accounting for >25% of the overall information) (Fig. 4.3-A). Within each cluster in Fig. 4.3-A, the proximity between adjacent points seems to be explained mostly by the sampling 'Year', suggesting that similar frequencies are observed for closer years however, the discrimination between the three clusters appears to have had the strong influence of 'District', spread vertically. Moreover, PCA seemed also to indicate a relationship between genotoxic damage and birds' survival, as well as between that and age (at a lower extent) (Fig. 4.3-B). PC1 was also strongly correlated to the variable 'Age', although to a lesser extent (compared to 'Year') (Fig. 4.3-B). In the same way, PC2 was also strongly correlated (positively) to 'Survival' (Fig. 4.3-B).

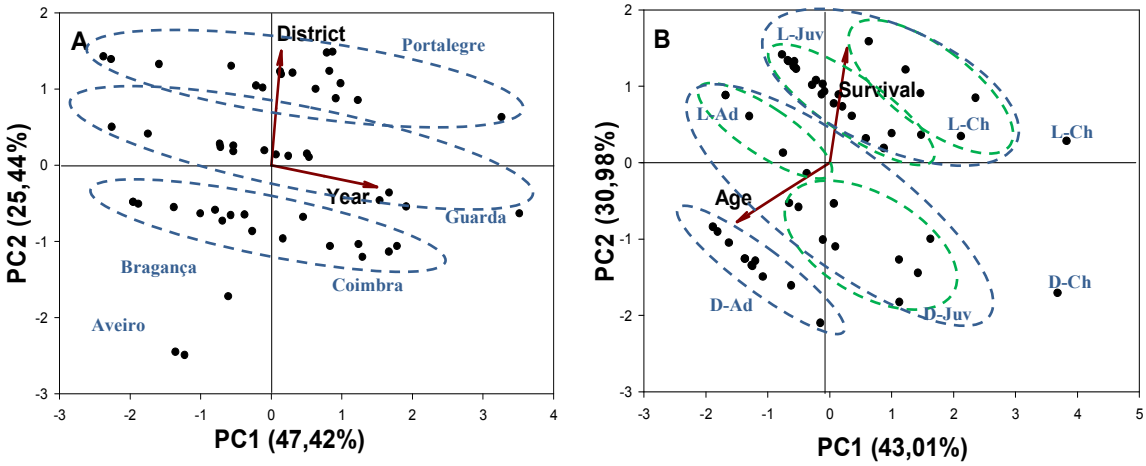

Fig. 4.3. Principal Component Analysis (PCA) of micronuclei (MN) and total nuclear abnormalities (TNA) in *Ciconia ciconia* with: (A) district and year, (B) survival and age, as explaining variables. N=52 individuals; Percentage of variability explained by each principal component is indicated in brackets for each axis; Dotted blue lines correspond to clusters identified graphically; Doted green lines within each cluster are only used to group points for identification porpoises; L-Chi= live chick, L-Juv=live juvenile, L-Ad=live adult, D-Ch=deceased chick, D-Juv=deceased juvenile, D-Ad= deceased adult.

TNA frequency was observed to increase in both species from 2007 to 2010 and decrease slightly in 2011, being these differences between years only statistically significant in *C. ciconia* (Fig. 4.4-A). Similarly, significant temporal differences were found to occur within different geographical sites, with individuals of *C. ciconia* from Coimbra and Portalegre following a similar increase and decrease pattern, except for Guarda in which no statistical differences were registered (Fig 4.4-B). Moreover, significant geographical differences were still found in 2011, with white stork *C. ciconia* population of

Coimbra showing a significant higher frequency of TNA than Portalegre's (Holm-Sidak, P<0.05).

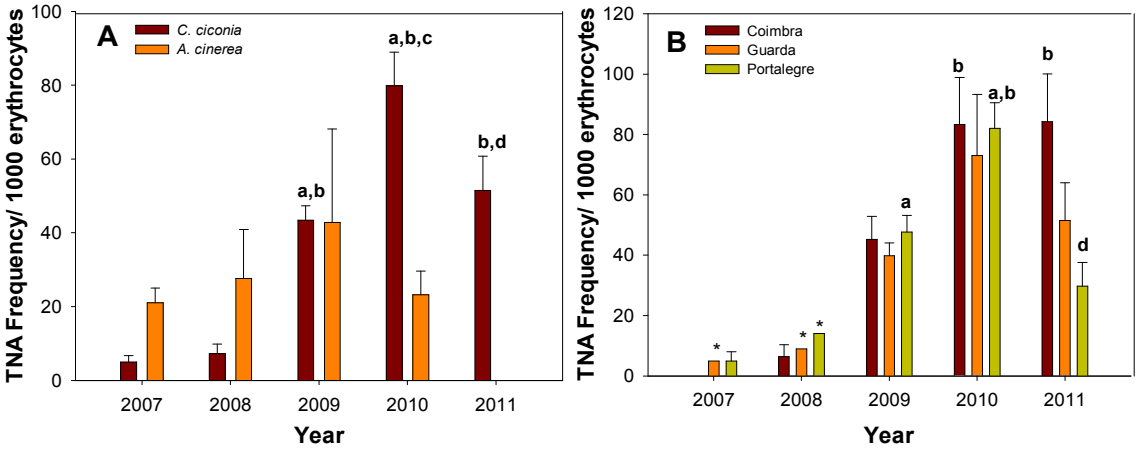

Fig. 4.4. Temporal variation of the TNA frequency in Portuguese populations of Ciconiiformes. (A)- *Ciconia ciconia* and *Ardea cinerea* variation in continental territory; (B)- District variation of TNA frequency in *Ciconia ciconia*. Significant differences (Holm-Sidak, P<0.05): a= different from 2007, b= different from 2008, c= different from 2009, d= different from 2010; *= sample size equal to 1.

MN frequency followed a similar pattern to TNA, with MN frequency generally increasing from 2007 to 2011 in *C. ciconia*, being this increase only statistically relevant in 2011 (Fig. 4.5-A). No defined pattern of MN variation was reported between years in *A. cinerea*, though. When analysing MN frequency within district, the same pattern of increase on MN frequency was observed to occur, but none of these differences were reported to be statistically significant.

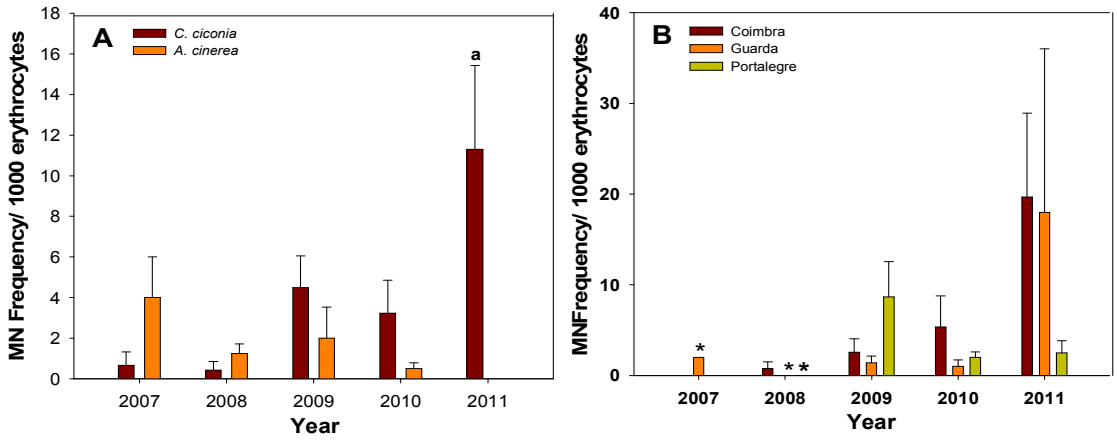

Fig. 4.5. Temporal variation of the MN frequency in Portuguese populations of Ciconiiformes. (A)- *Ciconia ciconia* and *Ardea cinerea* variation in continental territory; (B)- District variation of TNA frequency in *C. ciconia*. a= significantly different from 2008 (Dunn's test, P<0.05); *= sample size equal to 1.

PCA analysis seemed to indicate a variability in genotoxic damage influenced by survival and age of the individuals (Fig. 4.6-C and D) in *A. cinerea*, with PC1 and PC2 accounting for over 81% of the variability in the dataset. PCA showed a segregation of points both vertically and horizontally as an effect of individual age and survival. No clear spatial or temporal trends were, however, shown by PCA analysis in *A. cinerea* (Fig. 4.6-A and B), with PC1 and PC2 accounting for over 74% of the variability.

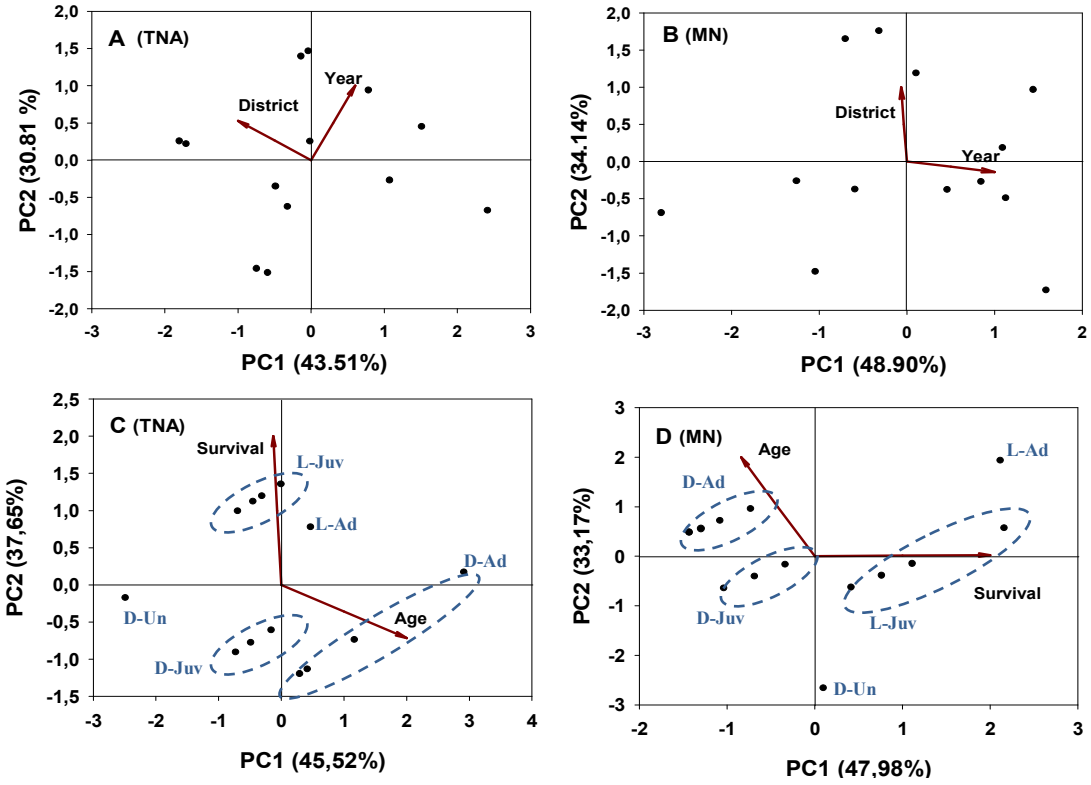

Fig. 4.6. Principal Component Analysis (PCA) of total nuclear abnormalities (TNA) and micronuclei (MN) in *Ardea cinerea* with: (A, B) district and year, (C, D) age and survival as explaining variables. N=13 individuals; Percentage of variability explained by each principal component is indicated in brackets for each axis; PCA analysis of TNA and MN frequencies are presented in separate because *Ardea cinerea* retained the highest variability; Dotted blue lines correspond to clusters identified graphically; L-Chi= live chick, L-Juv=live juvenile, L-Ad=live adult, D-Ch=deceased chick, D-Juv=deceased juvenile, D-Ad= deceased adult.

For both *C. ciconia* and *A. cinerea* an increase of DNA damage was observed with reduced survival of individuals (Fig. 4.3-B, Fig. 4.6-C,D), nonetheless for both species these differences were not statistically significant. Significant differences in the amount of DNA damage associated with age of individuals' were observed in *C. ciconia*, with chicks exhibiting a significantly higher frequency of MN than adults (Dunn's Test, $P<0.05$) and a higher frequency of TNA than juveniles (Dunn's Test, $P<0.05$). No significant differences

related to birds' age were reported in *A. cinerea*. Datasets of *A. purpurea* and *E. Garzetta* were too small to allow analysis of MN and TNA variation.

4.4.3. Genotoxic recovery following bird rehabilitation

DNA damage during bird rehabilitation was monitored in *C. ciconia*, *A. cinerea* and *A. purpurea* and it is presented in Fig. 4.7. In all case studies, MN frequencies of individuals during rehabilitation were found to decrease and stabilize within the interval of variation considered as normal for healthy birds. Regarding TNA frequency, in half of the cases studied total anomalies were found to decrease over time. A reversed tendency was, although, reported for the other half of the study cases with TNA frequency unexpectedly increasing over time but never exceeding 4.5% of damaged nuclei.

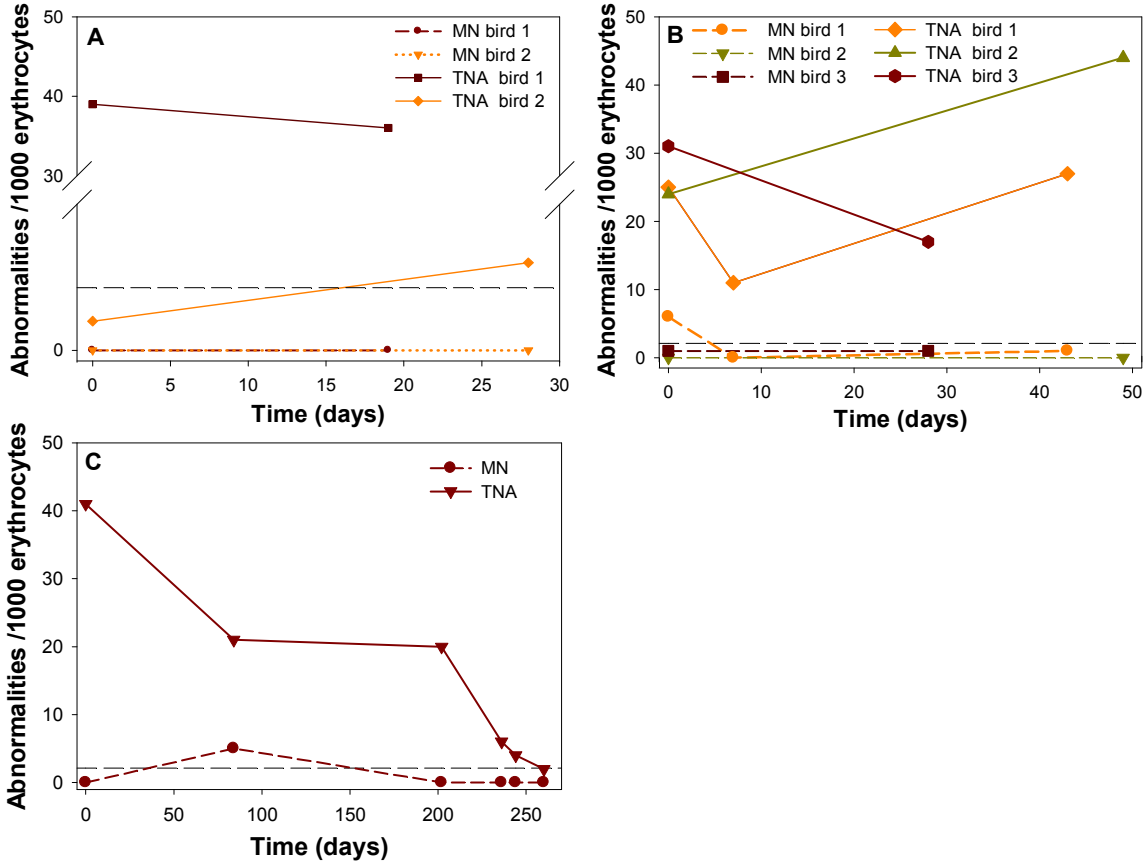

Fig. 4.7. Number of micronuclei (MN) and total nuclear abnormalities (TNA) variation over time per sampled individual of: (A) *Ciconia ciconia*, (B) *Ardea cinerea* and (C) *Ardea purpurea*. Dashed black lines correspond to the maximum normal level of MN for healthy individuals (2.14 MN/1000 erythrocytes) reported for Ciconiiformes and other avian species in the literature [6,11,12].

4.5 Discussion

Evaluation of damage induced in genetic material of erythrocytes has been widely used to detect exposure to genotoxicant compounds on fish, mammals and birds [6,13-16]. Studying the occurrence of these compounds is of most importance as DNA-damage may impair individuals' survival and reproductive success, thus altering population dynamics which may trigger a cascade of effects upon an ecosystems biota, and representing a threat to the ecosystem's equilibrium [2,4]. Methodologies used to monitor avian exposure to genotoxic compounds generally have relied on the analysis of micronucleus frequency or the comet assay. In the present study it was assessed the frequency of micronucleus (MN) and other nuclear aberrations in peripheral blood erythrocytes of Ciconiiformes.

In all species, micronuclei (MN) and other nuclear abnormalities were observed. In 23 cases studied, MN frequency was reported to be above the normal level of MN for healthy birds reviewed in the literature [6,11,12]. This could probably indicate that Portuguese populations of Ciconiiformes could be exposed to elevated concentrations of genotoxic chemicals on the environment, which could impair their health and modify population dynamics.

Total nuclear abnormalities (TNA) frequency was significantly higher in *C. ciconia* and *E. garzetta* than in *A. purpurea* and *A. cinerea*, indicating that diet preferences' could probably play an important role in Ciconiiformes' risk of exposure to genotoxicant compounds. Contrarily to the grey heron (*A. cinerea*) and the purple heron (*A. purpurea*) which are piscivorous, the white stork (*C. ciconia*) and the little egret (*E. garzetta*) are generalists, feeding opportunistically on a wide variety of both aquatic and terrestrial organisms (e.g. insects, earthworms, and amphibians) [17], thus frequenting a wider diversity of ecological niches. Consequently, this foraging behaviour could lead to an increased risk of exposure to environmental contaminants, including genotoxic compounds, which could possibly account for the significant increase of genotoxic damage observed in *C. ciconia* and *E. garzetta*.

When the correlation between the different categories of lesions scored and TNA frequency was tested, a weak correlation (*C. ciconia*) or no correlation at all (*A. cinerea* and *A. purpurea*) between MN and TNA frequencies was reported (Table 4.2). This may be indicative that formation of MN could be dependent of other factors rather than only genotoxic exposure (for example, it could be dependent of the degree of exposure), thus leading us to question: could the single use of MN frequency contribute to mask genotoxic effects on Ciconiiformes' populations? The answer to this question seems to us

dependent of whether we look or not at the genotoxic damage as a whole, independently of the severity of effects inherent to each type of analysis. TNA frequency may allow a more sensible measure of genotoxic damage than MN frequency, nonetheless the picture changes if we want to take into consideration the magnitude/severity of genotoxic exposure. MN formation has been used as an indicator of chromosome breakage and/or loss, which has been directly associated with an increase of cancer risk and/or aging [18]. Although the mechanisms underlying nuclear abnormalities formation are not yet fully understood, these lesions have been interpreted as analogous to MN [19], but with a lesser degree of severity. Relating to this point, it seems to us logical to conclude that in order to assess genotoxic exposure in Ciconiiformes' populations, the use of ENA assay may contribute to increase overall sensitivity of genotoxic assessment; nonetheless, interpretation of data should always take into account the distinct effect magnitudes inherent to MN and other nuclear abnormalities.

The second objective of this work was inferring whether it existed or not underlying patterns of genotoxic variation on Portuguese populations of Ciconiiformes. Generally, a temporal (yearly) increase in the frequency of both TNA and MN frequencies were observed in both *C. ciconia* and *A. cinerea* (Figs. 4.4 and 4.5), nevertheless these differences were only reported to be statistically significant in TNA frequency of *C. ciconia* (Fig. 4.4). This may be indicative of an increasing environmental exposure of individuals to genotoxic contaminants, with a peak in 2010. This same trend was consistently repeated when reducing margin of analysis at the district level, with individuals of *C. ciconia* from the districts of Coimbra and Portalegre evidencing also significant yearly increases in TNA frequencies registered (Fig. 4.4-B).

Spatial differences were also reported in *C. ciconia* with Coimbra's population exhibiting TNA values significantly higher than in Portalegre's population on the year of 2011. Additionally to this, Coimbra was also the district that registered a more steady increase of TNA and MN frequencies, not declining in 2011 as observed in Guarda and Portalegre, which may indicate that Ciconiiformes' populations inhabiting in this particular area could be exposed to higher levels of genotoxic compounds. The close location of rice fields and the characteristic pesticide existence nearby these areas could be a reason that justifies this pattern, as a common behaviour of Ciconiiformes, namely *C. ciconia*, is to forage into agricultural ponds; moreover, storks are also known to nest all around the rice field's area [20,21]. Increased genotoxic damage as a cause of pesticide exposure has been previously reported in the literature [22]. Although no recent statistical data regarding the amount of production of rice fields located in Coimbra is available, historically the

basin of river Mondego has been accounted as one of the most important areas of rice production in Portugal [23].

The intensive use of land by agriculture could have also promoted leaching of contaminants into water courses, thus increasing contaminant concentration. Moreover differences observed within cities could be related to the higher human population density of Coimbra, comparatively to Portalegre's, as high population densities would most likely increase the disposal of contaminants (including genotoxicant chemicals) into the environment, thus increasing the risk of exposure of local populations of white stork. These variations in TNA and MN frequency between years and districts in *C. ciconia* was further evidenced by PCA analysis (Fig. 4.3-A), which showed a segregation of points into three main clusters discriminated vertically by the influence of "District" and horizontally by 'Year', suggesting that within each cluster similar frequencies were most likely observed for closer years.

In both *C. ciconia* and *A. cinerea*, PCA analysis also seemed to indicate a relationship between genotoxic damage and birds' survival, as well as between that and age (Figs. 4.3. and 4.6). An increase of DNA damage was observed with reduced survival of individuals, this was further evidenced in PCA analysis, with survival of individuals' being clearly an important factor in induced MN and TNA frequencies on both species (Fig. 4.3-B, Fig. 4.6-C,D). Despite this graphical tendency, for both species these differences were not statistically significant. This absence of statistical significance could be, however, result of the high heterogeneity of individuals' sampled, thus making it difficult to detect through statistical analysis any underlying pattern.

DNA damage (MN and TNA) was observed to be higher in chicks of *C. ciconia* than in juveniles or adults. In two consecutive reports of baseline levels of MN on vertebrates, a similar trend was detected in a wide number of mammal species such as the dog (*Canis familiaries*), gray squirrel (*Sciurus aureogaster*) and the white-tailed deer (*Odocoileus virginianus*) [11,12]. Similarly, DNA damage was observed to decrease significantly with age, being MN frequencies higher in younger individuals than in adults. As hypothesized by the authors, these differences could be due to the inability of cellular mechanisms of chicks to cope with DNA damage as efficiently as adults or juveniles [11,12]. More specifically, these differences could be related of the fact that the reticuloendothelial system, which is involved in the removal of old and damaged erythrocytes from the blood, matures with age [11]. Thus, younger individuals of *C. ciconia* could exhibit similar higher rates of DNA damage due to their reduced ability to eliminate damaged erythrocytes.

The final aim of this work was to assess genotoxic recovery following bird rehabilitation. In order to achieve that, TNA and MN evolution over time of rehabilitation was monitored in two individuals of *C. ciconia*, three individuals of *A. cinerea* and one individual of *A. purpurea*. In all cases, MN frequency of individuals during rehabilitation was found to decrease and stabilize within the interval of 0 to 2.14 MN/1000 erythrocytes assumed as normal for non-exposed birds [6,11,12]. In half of the cases TNA frequency did not show a clear decreasing pattern during rehabilitation, with TNA frequency decreasing for example in *A. purpurea* and increasing over time in *A. cinerea,* although never exceeding 4,5% of damaged nuclei. All birds during their rehabilitation were maintained in a controlled environment, thus making it unlikely that such effects could be related to contamination of their surrounding environment, nonetheless exposure of individuals to genotoxic chemicals could have occurred through food items provisioned. Sporadic exposure of individuals to genotoxic contaminants through food ingested could be, therefore, one reason to account for such irregular pattern of variation on TNA frequency. This study was, to our knowledge, the first to perform the ENA assay in erythrocytes of avian species, thus no previous reports of TNA frequencies in non-exposed individuals are available in the literature to make a comparison and to confirm whether or not these birds had abnormal levels of TNA frequency. It should not be excluded therefore that, rather than an effect of exposure to genotoxicant substances, the high TNA frequencies observed during rehabilitation of individuals could be a consequence of DNA defects generated spontaneously.

In conclusion, spatial and geographical differences were detected in Portuguese populations of Ciconiiformes. TNA frequencies of *C. ciconia* were observed to increase significantly with year and, in 2011, vary spatially. Age was also found to be an important factor in both TNA and MN frequency, increasing with decreasing age of individuals of *C. ciconia*. These results suggest that the assessment of TNA, rather than only MN frequencies, may be a useful complementary tool for the study of induced genotoxicity in birds as it allows a more insightful and discriminating analysis of the genotoxic patterns underlying in populations of wild birds.

4.6 Acknowledgements

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4.7 References

1. Shugart LR, Theodorakis CW, Bickham AM, Bickham JW (2003) Genetic Effects of Contaminant Exposure and Potential Impacts on Animal Populations. In: Hoffman D, Rattner B, Burton GA, Cairns J, editors. Handbook of Ecotoxicology. Second edition ed. Boca Raton (FL): Lewis Publishers. pp. 1129-1147.

2. Baos R, Jovani R, Pastor N, Tella JL, Jimenez B, et al. (2006) Evaluation of genotoxic effects of heavy metals and arsenic in wild nestling white storks (Ciconia ciconia) and black kites (Milvus migrans) from southwestern Spain after a mining accident. Environmental Toxicology and Chemistry 25: 2794-2803.

3. Pastor N, Lopez-Lazaro M, Tella JL, Baos R, Hiraldo F, et al. (2001) Assessment of genotoxic damage by the comet assay in white storks (Ciconia ciconia) after the Donana Ecological Disaster. Mutagenesis 16: 219-223.

4. Costa PM, Neuparth TS, Caeiro S, Lobo J, Martins M, et al. (2011) Assessment of the genotoxic potential of contaminated estuarine sediments in fish peripheral blood: Laboratory versus in situ studies. Environmental Research: 25-36.

5. Kleinjans JCS, van Schooten F-J (2002) Ecogenotoxicology: the evolving field. Environmental Toxicology and Pharmacology 11: 173-179.

6. Quiros L, Ruiz X, Sanpera C, Jover L, Pina B (2008) Analysis of micronucleated erythrocytes in heron nestlings from reference and impacted sites in the Ebro basin (NE Spain). Environmental Pollution 155: 81-87.

7. Pacheco M, Santos MA (1997) Induction of EROD activity and genotoxic effects by polycyclic aromatic hydrocarbons and resin acids on the juvenile eel (Anguilla anguilla L.). Ecotoxicology and Environmental Safety 38: 252-259.

8. Stolen ED, Breininger DR, Frederick PC (2005) Using waterbirds as indicators in estuarine systems: successes and perils. Estuarine Indicators: 409-422.

9. Rodnan GP, Ebaugh FG, Fox MRS (1957) The Life Span of the Red Blood Cell and the Red Blood Cell Volume in the Chicken, Pigeon and Duck as Estimated by the Use of Na2cr51o4 - with Observations on Red Cell Turnover Rate in the Mammal, Bird and Reptile. Blood 12: 355-366.

10. Pacheco M, Santos MA (1996) Induction of micronuclei and nuclear abnormalities in the erythrocytes of Anguilla anguilla L exposed either to cyclophosphamide or to Bleached Kraft Pulp Mill Effluent. Fresenius Environmental Bulletin 5: 746-751.

11. Zuniga-Gonzalez G, Torres-Bugarin O, Luna-Aguirre J, Gonzalez-Rodriguez A, Zamora-Perez A, et al. (2000) Spontaneous micronuclei in peripheral blood erythrocytes from 54 animal species (mammals, reptiles and birds): Part two. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 467: 99-103.

12. Zuniga-Gonzalez G, Torres-Bugarin O, Zamora-Perez A, Gomez-Meda BC, Ibarra MLR, et al. (2001) Differences in the number of micronucleated erythrocytes among young and adult animals including humans - Spontaneous micronuclei in

43 species. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 494: 161-167.

13. Monteiro V, Cavalcante DGSM, Vilela MBFA, Sofia SH, Martinez CBR (2011) In vivo and in vitro exposures for the evaluation of the genotoxic effects of lead on the Neotropical freshwater fish Prochilodus lineatus. Aquatic Toxicology 104: 291-298.

14. Domingues I, Oliveira R, Lourenço J, Grisolia CK, Mendo S, et al. (2010) Biomarkers as a tool to assess effects of chromium (VI): comparison of responses in zebrafish early life stages and adults. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 152: 338-345.

15. Reichert WL, French BL, Stein JE (1999) Exposure of Marine Mammals to Genotoxic Environmental Contaminants: Application of the 32P-Postlabeling Assay for Measuring DNA-Xenobiotic Adducts<sup>＃</sup&gt. Environmental Monitoring and Assessment 56: 225-239.

16. Yauk CL, Fox GA, McCarry BE, Quinn JS (2000) Induced minisatellite germline mutations in herring gulls (Larus argentatus) living near steel mills. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis 452: 211-218.

17. IUCN (2012) IUCN Red List of Threatened Species [Online]. Available: http://www.iucnredlist.org/ [Accessed 5 July 2012].

18. Fenech M (2000) The in vitro micronucleus technique. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis 455: 81-95.

19. Oliveira M, Ahmad I, Maria VL, Ferreira CSS, Serafim A, et al. (2010) Evaluation of oxidative DNA lesions in plasma and nuclear abnormalities in erythrocytes of wild fish (Liza aurata) as an integrated approach to genotoxicity assessment. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 703: 83-89.

20. Hoyo J, Elliot A, Sargatal J, others a (1992) Handbook of the Birds of the World. Volume 1- Ostrich to Ducks. Barcelona: Lynx Edicions.

21. Cramp S, Simmons KEL, Brooks DJ, Collar NJ, Dunn E, et al. (1978) Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palaearctic. ; Cramp S, editor. Oxford: Oxford University Press

22. Bajpayee M, Pandey AK, Zaidi S, Musarrat J, Parmar D, et al. (2006) DNA damage and mutagenicity induced by endosulfan and its metabolites. Environmental and Molecular Mutagenesis 47: 682-692.

23. Lima A Current situation of rice production in Portugal and the main diseases that occur. Cahiers Option Méditerranéennes 15: 29-40.

Chapter 5

General Discussion and Conclusions

5.1 General Discussion and Conclusions

Birds have been used as bioindicators of environmental quality in a broad range of ecosystems [1-3]. They are conspicuous, very abundant, functionally important components of ecosystems and highly sensitive to environmental contaminants, thus being ideal species to monitor on field [1,3,4].

Over the past years, little attention has been given to the study of contaminants effects upon Portuguese populations of birds. The present study was an attempt to bridge the gap of data relative to risk assessment of environmental contaminant exposure on Portuguese birds, in particular of birds allowed for rehabilitation in wildlife recovery centres. More specifically, this study intended to clarify several aspects, namely whether or not Portuguese populations of birds are exposed on field to harmful levels of environmental contamination and how could these contamination lead to birds' illness and/or influence their rehabilitation.

In order to comprehend these issues, two types of biomarkers were assessed: cholinesterase (ChE) activity and micronuclei/ other nuclear abnormalities frequencies. ChE activity was measured and characterised in the plasma of three native bird species (*Ciconia ciconia*, *Ardea cinerea* and *Morus bassanus*) for further use in future biomonitoring studies, and measured in the brain of *Larus michahellis*, in order to assess possible neurotoxic effects related to pesticide exposure. Frequency of micronuclei (MN) and other nuclear abnormalities was used to monitor environmental exposure to genotoxicant compounds in *Ciconia ciconia*, *Ardea cinerea*, *Ardea purpurea* and *Egretta garzetta*.

The use of both ChE activity and MN/total nuclear abnormalities (TNA) frequencies showed to be in future an indicator of potential exposure of birds to environmental contaminants. For example, reactivation assay in brain of the seagull *L. michahellis* retrieved significant increases in ChE activity, suggesting that deceased individuals could have been exposed to harmful concentrations carbamate (CB) and organophosphorous (OP) pesticides, thus allowing to identify a possible scenario of acute poisoning by CB and OP compounds. Reactivation of ChE activity was based on the principle that chemical and spontaneous reactivation assays can be used to reactivate OP or CB-inhibited ChE, respectively [5]. Therefore, not only it was possible to relate cause of death with percentage of brain inhibition but, in addition, it was also possible to infer the class of pesticide involved on the incident reported. This type of approach proved to be, therefore, particularly insightful to screen for a possible pesticide poisoning. Nonetheless, further

knowledge on the type of compounds involved should always be confirmed by chemical analysis.

Elevated levels of genotoxic damage were observed in the Ciconiiformes studied, exceeding inclusively, in 23 cases, the level of 2.14 MN/1000 erythrocytes reviewed in the literature as normal for non-exposed birds [6-8]. In addition, analysis of MN and TNA were also observed to vary significantly ($P<0.05$) according to age, feeding habits, year of sampling and spatial origin of Ciconiiformes. These results suggest that Portuguese birds might be exposed on the environment to contaminants, which depending on the severity of exposure, may trigger the disruption of normal physiological processes on individuals. Eventually, these changes may generate a cascade of effects upon birds, leading to increased fragility of individuals, illness and ultimately death. For example, in a case of OP and/or CB exposure, ChE activity is inhibited causing the disruption of cholinergic function. This leads to a wide range of effects on birds, such as diarrhoea, ataxia and muscular weakness. Muscular weakness, for instance, could reduce ability of individual to find and catch food, thus reducing body condition and increasing fragility. With increasing severity of exposure, toxic effects may as well evolve, causing more acute effects as dyspnea and ultimately death. Seagulls studied exhibited, prior to death, similar effects with clear signs of muscular ataxia, diarrhoea and in some cases reduced body condition, reason why OP/CB poisoning was suspected and later corroborated by ChE reactivation techniques.

If it is easily recognizable the role that contaminants may have on birds' health, the same is not true for the effect that previous background exposures may have on birds' rehabilitation. TNA and MN frequency, for example, could not clarify whether survival of individuals could significantly relate to any extent with amount of DNA damage. This absence of consistent pattern could be, for instance, a consequence of the elevated heterogeneity of individuals studied (e.g. dissimilar ages, gender) that could be masking any underlying relation. On the other hand, this absence of relation may also be due to the fact that survival of individuals could be related to the type and magnitude of other injuries rather than previous contaminant exposure on field. In other words, and providing one example, a bird with a severe head trauma due to a collision against a pole of high voltage would most likely die due the trauma itself rather than as an effect of previous exposure to a genotoxic compound. Nonetheless, the severity of this head trauma could also have been magnified due to previous exposure to contaminants, as for example neurotoxic compounds, which probably would not be identified through a regular necropsy of the individual. Moreover, in this hypothetical scenario, inability of individual to move

away from the obstacle could also have been triggered by a neurologic dysfunction caused by this exposure, which without proper toxicological screening would probably be unnoticed.

An alternate way to evaluate contaminant effects upon recovery of birds' at wildlife centres could be directing this issue towards the study of more specific health-markers such as of immune function as this could more directly affect individual recovering (e.g. a bird with a bacterial infection would most likely have his chances of rehabilitation reduced if its immune function was compromised). Exposure to organochlorine compounds, for example, has been observed to cause misregulation of immune system of the black-footed albatross (*Phoebastria nigripes*) by lymphocyte proliferation which could lead to hypersensitivity [9]. Studying immune function responses could be, therefore, a good marker to assess whether background contaminant exposure could affect and/or be impairing at a significant scale birds' physiology and recovering.

It has been acknowledged that Portuguese birds are exposed on the environment to contaminants, in some cases at concentrations high enough to produce harmful effects such as illness of individuals and even death. It is unclear, however, at what extent background exposure of individuals to contamination could increase risk of mortality on birds with impaired health conditions unrelated to contaminant exposure (e.g. bacterial infection), nonetheless increased death probability due to impaired immunological responses could be a subject to explore. Considering this, could then ecotoxicological tools help to monitor and aid bird's recovery?

The use of ecotoxicological tools, in particular the ones used in this study (ChE activity and MN/TNA frequency), allowed to detect effects on birds studied consistent with exposure to environmental contamination, in some cases allowing to distinguish possible geographical variation (MN and TNA study) and class of contaminant (reactivation of ChE study). Using these biomarkers or similar ones could help to monitor previous background exposure of birds accepted for rehabilitation. This is of most importance, as such data in some cases could be crucial to detect early physiological signs of toxicity on individuals injured. Furthermore, this information would enable a more insightful evaluation of birds' health condition, thus contributing to a more adjusted and successful treatment.

5.1 References

1. Mistry J, Berardi A, Simpson M (2008) Birds as indicators of wetland status and change in the North Rupununi, Guyana. Biodiversity and Conservation 17: 2383-2409.

2. Goldstein MI, Lacher TE, Zaccagnini ME, Parker ML, Hooper MJ (1999) Monitoring and Assessment of Swainson's Hawks in Argentina Following Restrictions on Monocrotophos Use, 1996–97. Ecotoxicology 8: 215-224.

3. Monteiro LR, Furness RW (1995) Seabirds as monitors of mercury in the marine environment. Water, Air, & Soil Pollution: 851-870.

4. Stolen ED, Breininger DR, Frederick PC (2005) Using waterbirds as indicators in estuarine systems: successes and perils. Estuarine Indicators: 409-422.

5. Martin AD, Norman G, Stanley PI, Westlake GE (1981) Use of Reactivation Techniques for the Differential-Diagnosis of Organo-Phosphorus and Carbamate Pesticide Poisoning in Birds. Bulletin of Environmental Contamination and Toxicology 26: 775-780.

6. Quiros L, Ruiz X, Sanpera C, Jover L, Pina B (2008) Analysis of micronucleated erythrocytes in heron nestlings from reference and impacted sites in the Ebro basin (NE Spain). Environmental Pollution 155: 81-87.

7. Zuniga-Gonzalez G, Torres-Bugarin O, Luna-Aguirre J, Gonzalez-Rodriguez A, Zamora-Perez A, et al. (2000) Spontaneous micronuclei in peripheral blood erythrocytes from 54 animal species (mammals, reptiles and birds): Part two. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 467: 99-103.

8. Zuniga-Gonzalez G, Torres-Bugarin O, Zamora-Perez A, Gomez-Meda BC, Ibarra MLR, et al. (2001) Differences in the number of micronucleated erythrocytes among young and adult animals including humans - Spontaneous micronuclei in 43 species. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 494: 161-167.

9. Finkelstein ME, Grasman KA, Croll DA, Tershy BR, Keitt BS, et al. (2007) Contaminant-associated alteration of immune function in black-footed albatross (Phoebastria nigripes), a North Pacific predator. Environmental Toxicology and Chemistry 26: 1896-1903.